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# **Stress Responses of the Marine Diatom**

## ***Skeletonema* spp. to Polyunsaturated**

### **Aldehydes (PUAs)**

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**Doctor of Philosophy in Biological Sciences**

**September 2012**

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## Abstract

Diatom-derived polyunsaturated aldehydes (PUAs) are secondary metabolites acting as teratogens against grazers, allelochemicals by inhibiting the growth of closely phytoplankton species, and also as signal molecules to determine the cell fate of diatom populations.

The work presented here focuses on Nitric Oxide (NO) and Reactive Oxygen Species (ROS) production in response to PUAs in *Skeletonema marinoi*, a cosmopolitan, bloom forming and PUA-producing diatom species. In the first part of the thesis I addressed the problem of whether PUA-producing species might have evolved different stress response mechanisms with respect to non-PUA producing ones and if this may underlie their different ecological success. *S. marinoi* was exposed to different PUAs: DECA, which is not produced by *S. marinoi*, OCTA and HEPTA, which are PUAs commonly produced by this diatom and a mixture of these last two (MIX). A reduction in NO production was observed in response to all PUAs tested, probably due to consumption of physiological levels of NO, possibly indicating that this messenger acts as a growth regulator under optimal growth conditions.

In the second part, a comparison with the non-PUA producing diatom *Phaeodactylum tricornutum* revealed different reactions to the same PUAs (i.e. DECA), with an increase in NO production in DECA-exposed *P. tricornutum*, whereas in response to OCTA a reduction in NO production was evident. Thus, NO production in response to PUAs appears to be both PUA-specific and species-specific. Additionally, *S. marinoi* cells exposed to the photoinhibitor DCMU presented an increase in NO production, indicating that NO production in *S. marinoi* is likely to be also stress specific.

In terms of ROS production, *S. marinoi* showed a sharp increase in ROS production upon exposure to all PUAs except for DECA that is not produced by this diatom. In addition, an enhanced synthesis of xanthophylls in OCTA-exposed *S. marinoi* cells was observed, likely to act as antioxidants against ROS production thereby assuring the maintenance of the photosynthetic performance at intermediate concentrations of PUAs.

Additionally, in the congeneric species *Skeletonema tropicum*, PUAs induced an overexpression of a gene coding for a death specific protein (*ScDSP*), thought to be involved in an autocatalytic-type of cell death, also linked to a concentration-dependent increase in ROS production. This points to a key role of ROSs in mediating the response to PUAs leading to resistance or cell death, as for instance during the final stages of blooms.

Altogether, the data show a differential reaction of cells to PUAs, depending on the diatom species, the PUA type and concentration and the stress factor applied. This suggests a role of PUAs as signal molecules at the cellular and population levels. This is likely to underlie the ecological differences and evolutionary success of PUA and non-PUA producing species.



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## Abbreviations

**2ME** 2-methoxyestradiol

**APX** Ascorbate peroxidase

**ATP** Adenosine triphosphate

**CAT** Catalase

**Chl** Chlorophyll

**CM-H<sub>2</sub>DCFDA** 5-6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester

**cPTIO** carboxy-PTIO

**CuFL** Copper fluorescein

**DAF-FM DA** 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate

**DCMU** Diuron 3-(3,4-Dichlorophenyl)-1,1-dimethylurea

**Dd** Diadinoxanthin

**DEANO** DEA NONOate

**DECA** 2E,4E/Z-decadienal

**DETC** sodium diethyldithiocarbamate trihydrate

**DHR123** Dihydrorodamine 123

**DMSO** dimethylsulfoxide

**DNAME** D-NAME-hydrochloride

**Dt** Diatoxanthin

**EC50** Half maximal effective concentration

**EPA** Eicosapentaenoic acid

**FAHs** Fatty acid hydroperoxides

**FCM** Flow cytometry

**Fm** Maximal fluorescence after dark acclimation

**FSW** Filtered sea water

**Fv** Variable fluorescence after dark acclimation

**GPX** Glutathione peroxidase

**GSH** Glutathione

**H<sub>2</sub>O<sub>2</sub>** Hydrogen Peroxide

**HEPTA** 2E,4E/Z-heptadienal

**LNAME** L-NAME-hydrochloride

**LOX** Lipoxygenase

**LSU** Large subunit

**MAA** Mycosporine-like amino acids

**MAPKs** Mitogen-activated protein kinases

**MeOH** Methanol

**MIX** Combination of OCTA and HEPTA

**mRNA** messenger RNA

**NADH** Nicotinamide Adenine Dinucleotide

**NADPH** Nicotinamide Adenine Dinucleotide Phosphate

**NO** Nitric Oxide

**NOS** Nitric Oxide Synthase

**NPQ** Non Photochemical Quenching

**NR** Nitrate Reductase

**O<sub>2</sub><sup>-</sup>** Superoxide Anion

**<sup>•</sup>OH** Hydroxyl Radical

**ONOO<sup>-</sup>** Peroxynitrite

**OCTA** 2E,4E/Z-octadienal

**PCD** Programmed Cell Death



**PE** Photosynthetic Efficiency

**PSI** Photosystem I

**PSII** Photosystem II

**PUAs** Polyunsaturated Aldehydes

**PUFAs** Polyunsaturated Fatty Acids

**PX** Peroxidase

**rDNA** Ribosomal DNA

**RNS** Reactive Nitrogen Species

**ROS** Reactive Oxygen Species

***ScDSP*** *Skeletonema costatum* Death Specific Protein

**SD** Standard Deviation

**SE** Standard Error

**SOD** Superoxide Dismutase

**ST** Sodium Tungstate

**Tempol** 4-Hydroxy-TEMPO

**TEP** Transparent Exopolymeric Particles

**UA** Uric Acid

**XC** Xanthophyll cycle

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# CHAPTER 1

## State of the Art and Thesis Aims

### 1.1 Introduction

#### 1.1.1 Marine Phytoplankton and the Role of Diatoms

*Phytoplankton* is a term introduced in 1897 that indicates a very diverse, polyphyletic group of mainly unicellular prokaryotic and eukaryotic photosynthetic organisms floating and drifting with the currents in marine and fresh waters (Falkowski and Raven, 1997). Initial attention to the possible role of phytoplankton as the base of the marine food web started with the physiologist Victor Hensen, who published his revolutionary thoughts in 1887 (Hensen, 1887; Smetacek, 1999). In his work Hensen first coined the term *plankton*, which originated from Homer's *Odyssey*, and with which he wanted to indicate random and purposeless drifting. His most original and innovative intuition was that plankton populations were acting as a link from the very small to the very large, which led him to elaborate the concept of 'metabolism of the sea' (Smetacek, 1999). In this view, organisms were interacting with each other, with primary producers taking up resources and fixing energy which would be transferred to the consumers. In his work, Hensen referred to phytoplankton as 'the blood of the sea' (Smetacek, 2012; Smetacek et al., 2002).

If Victor Hensen was the pioneer of the conceptual ground-breaking concept of the potential role of (phyto) planktonic organisms in marine food webs, his contemporary, the well-known naturalist, Ernst Haeckel had the great merit to make such marine



microscopic life popular through his notable graphical work (Fig. I-1) (Breidbach, 2005; Haeckel, 1904). However, differently from Hensen, Haeckel seemed to believe that phytoplankton organisms were beautiful but not significant from an ecological point of view, and he indicated them as 'philosophical dirt'. Since then, our understanding and view has dramatically changed. We now know that although representing less than 1% of Earth's photosynthetic biomass, marine phytoplankton account for more than 45% of the photosynthetic net primary production on Earth, being responsible for the majority of the flux of organic matter transferred to higher trophic levels and fuelling ocean biogeochemical cycles (Field et al., 1998).

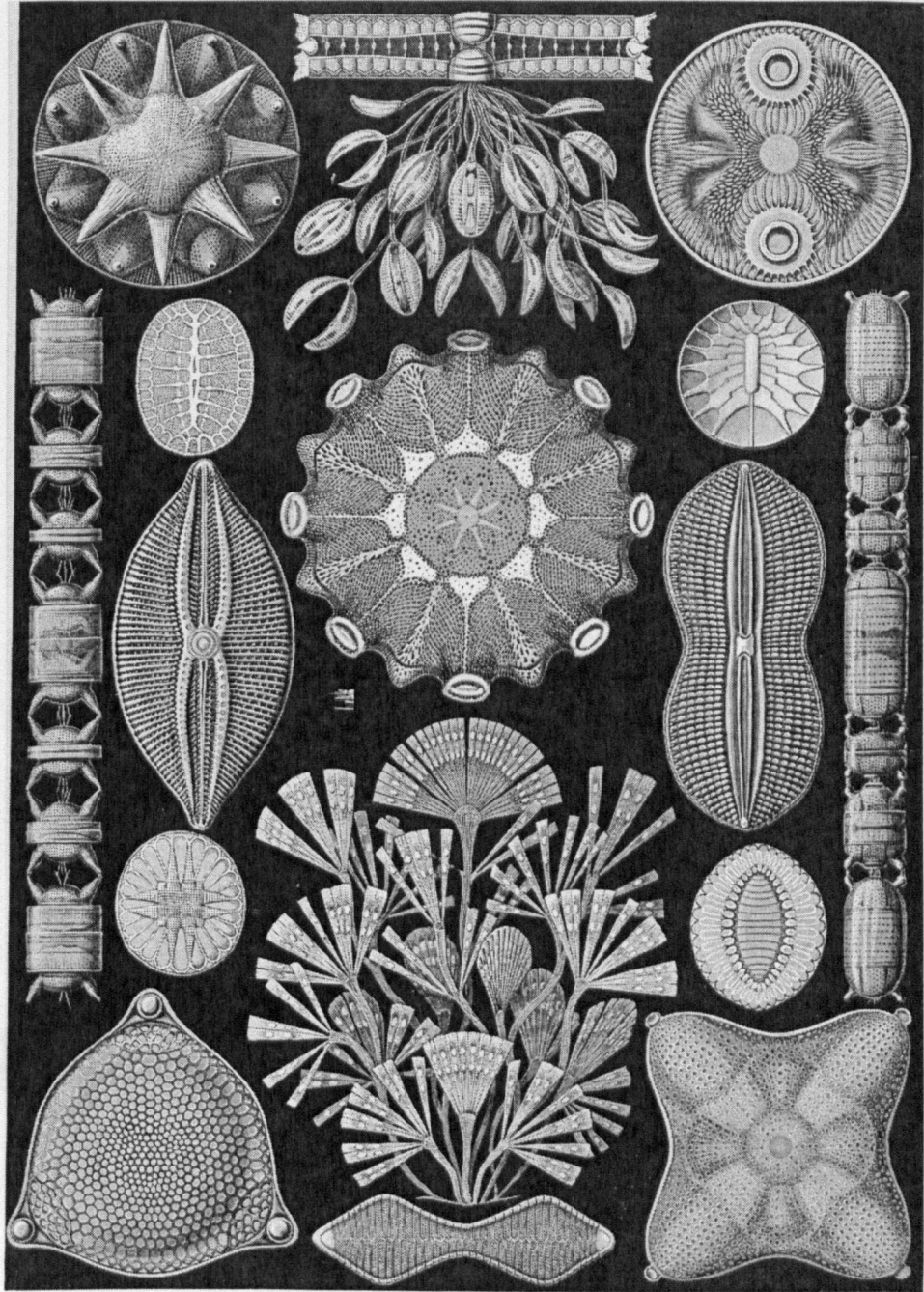


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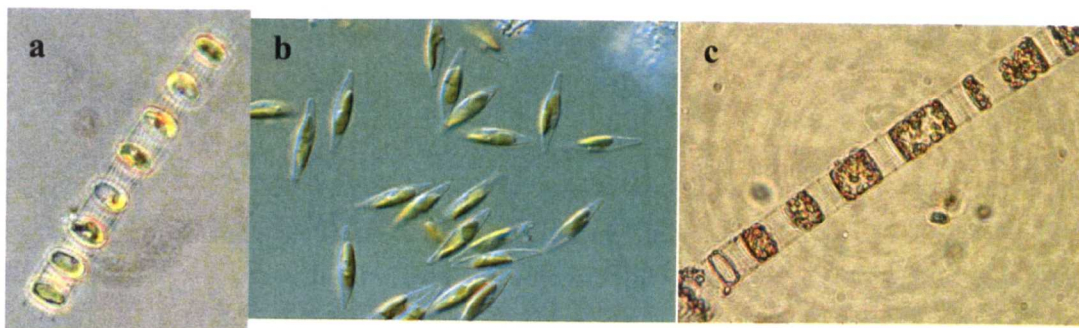


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Cyanobacteria are the only prokaryotic members of phytoplankton; the other, eukaryotic components include diatoms, dinoflagellates, coccolithophores and green algae (Reynolds, 2006). Approximately 40 % of all marine phytoplankton species are diatoms, and they are known to have a key ecological and biogeochemical role, in particular in nutrient-rich systems (Falkowski et al., 2004). It has been estimated that diatoms are responsible for about one-fifth of the photosynthesis on Earth (Nelson et al., 1995). The word diatoms derives from the Greek *diatomos*, which means ‘cut in half’, referring to their peculiar feature of two-part cell walls consisting of biogenic silica. When compared to terrestrial plants, diatom evolutionary history is far more complex (Parker et al., 2008). During the course of evolution, oxygenic photosynthesis seems to have evolved only once, subsequently spreading through endosymbiosis into different eukaryotic clades (Delwiche, 1999; Palmer, 2003). 1.5 billion of years ago, the initial, primary endosymbiosis took place, when a cyanobacterium was incorporated into a eukaryotic heterotrophic cell, giving rise to the photosynthetic plastids of the plants, the red and the green algae (Yoon et al., 2004). Around 500 million years later, a secondary endosymbiosis event occurred (Gibbs, 1981; Parker et al., 2008). Different eukaryotic cells incorporated the red primary symbiont, which was transformed into the plastids of a group of different photosynthetic organisms (collectively termed Stramenopiles), among which appeared the diatoms (Armbrust, 2009; Falkowski et al., 2004). The reason why multiple endosymbiotic events occurred during the course of evolution is still unclear. However, it is believed to be the consequence of a whole ocean denitrification (e.g. anoxic events), during which a lack of nitrogen might have supported a strong selection pressure for the development of new endosymbiotic events (Falkowski et al., 2004).

Given the importance of diatoms in marine ecosystems both from an evolutionary and contemporary point of view, diatom genomes are fundamental for understanding diatom evolutionary innovations and the peculiar features characterizing their metabolism (Bowler et al., 2010). So far, the complete genome sequences of two diatoms have been obtained, that of the centric diatom *Thalassiosira pseudonana* (Armbrust et al., 2004) and that of the pennate diatom *Phaeodactylum tricornutum* (Bowler et al., 2008). It has been shown that only about 50% of the identified genes within their genomes could be assigned a putative function based on our current knowledge, and that about 35% are diatom-specific (Bowler et al., 2008). Additionally, it was found that different bacterial genes were present within both diatom genomes (Bowler et al., 2008). In particular, *P. tricornutum* genome has been reported to contain at least 587 bacterial genes, which represent approximately 5% of its total gene content. Moreover, more than half of these genes have also been shown to be present in *T. pseudonana*, confirming that the transfer of these genes dates back to early evolutionary times. This is of particular importance considering that horizontal gene transfer is still considered uncommon in eukaryotes with respect to bacteria and archaea (Keeling and Palmer, 2008). The use of diatom genome sequences is particularly important for shedding light on diatom metabolic potential and for improving our understanding of their role in global biogeochemical cycles (Bowler et al., 2010).

A type of approach that has been proven useful and that should be implemented is, in fact, to study gene expression in response to different stimuli of ecological relevance, which has been done for both sequenced diatoms (Allen et al., 2008; Mock et al., 2008). However, from an ecological point of view, such an approach should also be implemented with a different range of ecologically-relevant and more representative diatom species, for which whole-genome studies will be required in the future.

Additionally, through the development of different molecular techniques (e.g. Siaut et al., 2007; De Riso et al., 2009), a key point to be now addressed is the assignment of specific roles to genes of unknown functions (Bowler et al., 2010). Another interesting aspect is represented by the recent findings that, within a single diatom species, genetically distinct populations with different physiological abilities exist (Rynearson and Armbrust, 2004; Rynearson et al., 2009; Casteleyn et al., 2010). In turn, genetically distinct strains can be present within individual populations (Rynearson and Armbrust, 2005). In this view, it becomes fundamental to consider that the sequencing, and, more in general, molecular studies of different diatom populations (of the same species) will provide new information about the process of evolution by natural selection (Rynearson and Palenik, 2011). Finally, a key challenge will be to put the molecular data back into the larger context of both cellular and eco-systems biology (Mock and Kirkham, 2012). In particular, in terms of ecosystems, an essential aspect to be considered is both environment-community interactions, as well as community-community interactions.

### **1.1.2 Chemical Ecology in Marine Ecosystems**

Chemical ecology is defined as “the relationship between the structure and function of metabolites and how these affect organisms in the environment, controlling the coexistence and coevolution of species” (Cembella, 2003). Chemical cues in general are believed to constitute the ‘language of life in the sea’ (Hay, 2009). Chemical mediated biotic interactions strongly affect population structure and community organization. In particular, a recent review by Hay (2009), nicely presented how chemical signals can act in regulating ecosystem functioning at different levels. Firstly, chemical metabolites from different marine organisms are known to be involved in the ecology of defence, i.e. acting to deter predators (e.g. Tillmann, 2004; Hay, 1996), pathogens (e.g. Gilturmes and

Fenical, 1992; Giltunes et al., 1989) as well as competitors (e.g. Gross, 2003; Legrand et al., 2003; Thacker et al., 1998). Secondly, chemical cues can also have stimulatory effects (as opposed to deterrent) for feeding (e.g. Coleman et al., 2007; Finelli et al., 2000) and reproduction (e.g. Stebbing et al., 2003; Sato et al., 2011; Kamio et al., 2002). Another interesting role proposed by Hay (2009), is that defined as ‘the smell of death’ attributable to some of these compounds, which can indirectly influence populations, community and ecosystems. In particular, chemically sensing the nearby death of an organism can have a strong influence on individual behaviours that can then have consequences on a larger-scale, e.g. during phytoplankton blooms and in changing trophic interactions (Peacor and Werner, 2001; Trussell et al., 2004; Byrnes et al., 2006; Long et al., 2007). Finally, chemical cues have also been shown to affect large-scale foraging, as in the case of dimethylsulfide (DMS) and seabirds (e.g. Nevitt, 2008), reef fishes (DeBose et al., 2008) and whale sharks (Martin, 2007), as well as habitat use, as in the case of salmon (e.g. Helfield and Naiman, 2001; Helfield and Naiman, 2002). These effects might in turn have a profound impact on nutrient and energy exchange both among very diverse communities and across different geographic scales, altering ecosystem structure and functioning (Hay, 2009).

Allelopathy is known to play a key role in interactions among different marine communities (e.g. Hulot and Huisman, 2004). The word allelopathy, mainly indicating chemical interference competition for resources, derives from the Greek *allelon* and *pathos*, meaning ‘to suffer reciprocally’. It has been proposed however, that besides negative effects, allelopathy could refer to both inhibitory and stimulatory interactions between and among different types of plants, also including effects on microorganisms (Molish, 1937). There are some questions about the possibility that such interactions could be active in natural marine populations, due to cell densities and the consequent

concentrations of specific allopathic molecules (e.g. Jonsson et al., 2009). However, allelopathy is believed to play a potentially important role in direct cell-to-cell interactions when conditions of high cell densities arise, when signalling processes could mediate recognition of substances and subsequent reactions (Mock and Kirkham, 2012).

Approximately two hundred species belonging to different phytoplankton groups such as diatoms, dinoflagellates, cyanobacteria and prymnesiophytes, are known or hypothesized to induce deleterious or beneficial effects on a broad range of different organisms (Landsberg, 2002). Many microalgae produce a number of different secondary metabolites, which are molecules that are not directly involved in basic cellular metabolism, and which are released into the environment (Hay, 1996). The biological role played by these compounds is often considered unclear. Compared to the longer studied and better understood chemical ecology interactions in terrestrial plants (Inderjit and Duke, 2003), the chemical ecology of marine phytoplankton has received less attention mainly due to technical difficulties in measuring interactions between physical, chemical and biological factors at different time and space scales (Wolfe, 2000). More attention has instead been given to chemical ecological interactions among invertebrate species (e.g. sponges, echinoderms and polychaetes), and in particular in benthic tropical ecosystems, where both species diversity and resource competition are expected to be high (Hay and Fenical, 1996). Nevertheless, there is increasing evidence that a number of different microalgal secondary metabolites may also play an important role for instance during algal bloom development and decay (e.g. Landsberg, 2002). Additionally, different studies indicate that phytoplankton-derived secondary metabolites might affect growth and physiological responses of bacteria and competitor



species (e.g. Legrand et al., 2003), and/or act as feeding deterrents against predation (e.g. Tillmann, 2004).

An important aspect to be considered when trying to understand the role of allelochemical interactions in general, is that multiple levels of interactions occur simultaneously. A recent study on the allelopathic dinoflagellate *Alexandrium tamarense* showed that the effects of algal compounds strongly depends on the initial structure of the biological community copresent with the dinoflagellate (Weissbach et al., 2011). Indeed, it has been proposed that using secondary metabolites for multiple purposes could be a strategy to improve efficiency by increasing the number of effects elicited by a single molecule (Wolfe, 2000).

### **1.1.3 Polyunsaturated Aldehydes (PUAs)**

Besides the above mentioned toxic metabolites, certain diatoms are also known to produce a peculiar class of toxic secondary metabolites belonging to the complex class of oxylipins, among which the best known and best characterized molecules are the teratogenic compounds known as polyunsaturated aldehydes (PUAs) (Pohnert, 2000; d'Ippolito et al., 2004; Cutignano et al., 2006). The term “oxylipins” was first introduced by Gerwick et al. (1991), to describe fatty acid-derived oxygenated compounds in marine algae. PUAs result from the oxidation of polyunsaturated fatty acids (PUFAs), and in diatoms PUAs are rapidly released upon loss of cell integrity, for example upon grazing by copepods (Pohnert, 2000), senescence or viral lysis. Upon cell membrane disruption lipase enzymes release PUFAs from complex lipids (Pohnert, 2005; d'Ippolito et al., 2004). After PUFAs are released, they are oxidized by lipoxygenase enzymes (LOXs) to fatty acid hydroperoxides (FAHs), which are then converted to PUAs by lyase activity (Pohnert, 2002; d'Ippolito et al., 2006).

Chloroplastic glycolipids are a source of C<sub>16</sub>-PUFAs, which are then converted to the C<sub>8</sub> aldehydes 2E,4E/Z,7Z-octatrienal and 2E,4E/Z-octadienal, respectively (d'Ippolito et al., 2003; d'Ippolito et al., 2004; Cutignano et al., 2006). The C<sub>20</sub>-PUFA eicosapentaenoic acid is instead released from membrane phospholipids and is the precursor of the C<sub>7</sub> aldehyde 2E,4E/Z-heptadienal and the C<sub>10</sub> aldehyde 2E,4E/Z,7Z-decatrienal. PUAs from marine diatoms were first isolated and identified by Miralto et al. (1999) from *Thalassiosira rotula*, *Skeletonema marinoi* and *Pseudo-nitzschia delicatissima*, as the two C<sub>10</sub> 2E,4E/Z-decadienal and 2E,4E/Z,7Z-decatrienal. PUAs in freshwater diatoms had been identified previously (Wendel and Juttner, 1996). Later on, a screening of 51 marine diatoms, including 71 isolates, revealed that PUAs production is widespread in different diatom species, and that it is species- and strain-dependent (Wichard et al., 2005a). In this last study, 38% of the investigated isolates resulted to be able to produce PUAs (Wichard et al., 2005a). Rapid production of oxylipins upon cell membrane disruption is probably dependent on the compartmentalization of enzymes and substrates, allowing release of free fatty acids as substrates for LOXs only when cell integrity is lost (Pohnert, 2005). The release of PUAs had been initially excluded for intact cells (Pohnert, 2002), and considered only as a mechanism of chemical defence against grazers; however, recently it has been observed that intact cells of *Skeletonema marinoi* release PUAs into the cell medium at the end of stationary phase before going into senescence independently from grazing (Vidoudez and Pohnert, 2008). Also, PUA production can be modulated by endogenous and external factors as growth stage and nutrient status (Ribalet et al., 2009; Ribalet et al., 2007b).

Among PUAs, 2E,4E-decadienal has become a model aldehyde to induce detrimental effects on the reproduction and development of several marine invertebrates, such as echinoderms, polychaetes, ascidians, crustaceans and molluscs (Caldwell, 2009;

Caldwell et al., 2003; Caldwell et al., 2002). Despite being the most used PUA in toxicological experiments, decadienal is not the most common PUA present in marine phytoplankton. In a survey of 51 species of marine diatoms, DECA was the least detected PUA (Wichard et al., 2005a) and this also appears to be the case at sea (Vidoudez et al., 2011b). Adolph et al. (2003) determined that the inhibitory activity of PUAs is dependent on the reactive Michael acceptor element; molecules belonging to this class are unstable and react with nucleophiles (i.e. they are attracted by positive nuclear charges), and they are thus often associated with toxicity due to protein modification (Refsgaard et al., 2000).

The bloom-forming diatom *Skeletonema marinoi* produces the C<sub>7</sub> aldehyde 2E,4E/Z-heptadienal, as well as the C<sub>8</sub> aldehydes 2E,4E/Z,7Z-octatrienal and 2E,4E/Z-octadienal (d'Ippolito et al., 2004), whereas it does not produce the C<sub>10</sub> 2E,4E/Z-decadienal (DECA). PUAs production in *Skeletonema marinoi* has been shown to be dependent on the age of the culture, going from 1.2 fmol cell<sup>-1</sup> in the exponential phase to 4.2 fmol cell<sup>-1</sup> in the stationary phase (Ribalet et al., 2007b). Additionally, PUAs production was found to increase during nutrient limitation, indicating that physiological conditions of the cells and nutrient stress represent active factors in regulating toxin production (Ribalet et al., 2007b). Thus Ribalet et al. (2007b) speculated that in the natural environment, during bloom events, nutrient limitation could act as a signal in driving cell death and the subsequent termination of the bloom itself. In diatoms, oxylipin production, such as PUAs, is known to be a continuous process which is reinitiated when the substrate is removed from the enzyme (Fontana et al., 2007a). At sea, due to PUAs being washed away by diffusion, it is likely that this steady state would be constantly altered, so PUA production would continue until

consumption of their precursor PUFAs. Such a mechanism would help maintain high local PUA concentrations at sea (Ribalet et al., 2007a).

Evidence is accumulating that, oxygenated fatty acid degradation products other than PUAs are also produced from fatty acid precursors, and that they can cause similar biological effects on grazers as those of PUAs (Fontana et al., 2007b). Fontana et al. (2007b) found that two species of the marine diatom *Chaetoceros*, which do not produce PUAs, were able to impair hatching success in the copepod *Calanus helgolandicus*. They found that *Chaetoceros* spp. were able to produce fatty acid hydroperoxides (FAHs) and oxylipins such as hydroxyacids (HEPEs) and epoxyalcohols (HepETEs), as well as highly reactive oxygen species (ROS). This would suggest that the observed effects of diatom diets is likely to be due not only to a single class of compounds (i.e. PUAs), but rather to a plethora of toxins to which grazers and other surrounding organisms are exposed either during feeding or for instance in the proximity of a diatom bloom (Ianora and Miralto, 2010). Some diatom species are known to produce both PUAs and other oxylipins [e.g. *Thalassiosira rotula* (d'Ippolito et al., 2005) and *S. marinoi* (Fontana et al., 2007b)], while other species have been shown to produce only metabolites other than PUAs [e.g. *P. delicatissima* (d'Ippolito et al., 2009), and *Chaetoceros socialis* and *C. affinis* (Fontana et al., 2007b)]. In particular, the hydroxyacid 15S-HEPE has been identified in the diatoms *P. delicatissima* (d'Ippolito et al., 2009) and *Skeletonema marinoi* (Fontana et al., 2007b). Indeed, Ianora et al. (2011b) were the first to test the biological activity of oxylipins other than PUAs on copepod reproduction. They found that 15S-HEPE was able to negatively affect both egg production and hatching success, providing an explanation as to why some diatoms negatively affect the reproductive success of copepods even if they do not produce PUAs (Wichard et al., 2008; Dutz et al., 2008). Additionally, the non-volatile oxylipins

hydroxyacids have been found to induce reproductive failure in the copepod *Temora stylifera* (Barreiro et al., 2011).

Traditionally, diatoms have been considered an ideal food for zooplankton larval growth and the consequent transfer of energy and organic carbon through the food chain (Ianora et al., 2006). However, starting from the early 1990's, evidence showing that marine diatoms could also have detrimental effects on other organisms has accumulated. In particular, Ban et al. (1997) introduced the so-called 'paradox of diatom-copepod interaction', based on the fact that copepods that fed on certain diatom species showed reduced fecundity and hatching success. Ianora and Poulet (1993) had already reported that a diatom diet of *Thalassiosira rotula* was responsible for low hatching success in copepods, even in the presence of high egg production. Similarly, Uye (1996) reported that copepods that had fed on diatoms generated deformed nauplii, which were not able to survive. Subsequently, Poulet et al. (1994) proposed that the reason for the negative effects of diatoms on copepod reproduction was due to microalgal anti-mitotic compounds that accumulate in the reproductive organs of copepods blocking embryogenesis. Indeed, in 1999, Miralto and co-workers were the first to identify diatom-derived PUAs as the toxic compounds responsible for these effects. These findings have been further confirmed by Pohnert (2000); Pohnert et al. (2002); Pohnert (2002); d'Ippolito et al. (2002a); d'Ippolito et al. (2003); d'Ippolito et al. (2002b), and Wichard et al. (2005a) were able to identify a more complete range of PUAs from different species of marine diatoms using different methodologies.

Miralto et al. (1999) not only were the first to isolate and identify diatom-derived PUAs, but also to demonstrate that these compounds block embryonic development of copepods and sea urchin embryos. Later, it was further demonstrated that PUAs act as grazing deterrents through an insidious-type of toxic mechanism, which does not stop

copepods from feeding, but rather impairs their recruitment, and restrains the cohort size of the next generation (Ianora et al., 2004). Substances that are able to induce congenital malformations in the offspring, leading to embryo or fetal mortality, are known as teratogens. Indeed, this was the first time that a teratogenic-like chemical defence had been demonstrated for unicellular algae (Ianora and Miralto, 2010). Since this first report, a number of different studies and reviews have addressed the effects of different diatoms on copepod reproduction (e.g. Paffenhofer et al., 2005; Ianora et al., 2003; Buttino et al., 2004; Paffenhofer, 2002). Interestingly, recent advances in this field have come from two pioneering studies looking, for the first time, at the effects of diatom diets on gene expression patterns in the copepod *Calanus helgolandicus* (Lauritano et al., 2011b; Lauritano et al., 2011a). In particular these authors showed that PUAs act by inhibiting tubulin-polymerization (Lauritano et al., 2011a) as well as primary defence systems involving genes such as aldehyde dehydrogenases and heat shock proteins (Lauritano et al., 2011b).

Besides the deleterious effects on copepods, inhibition of embryonic development and fertilization in response to PUAs has been shown in different marine invertebrates, including polychaetes and echinoderms (Caldwell et al., 2002), sea urchin embryos (Romano et al., 2003), shrimps (Caldwell et al., 2003; Zupo et al., 2007), rotifers (Taylor et al., 2005), and the freshwater cladoceran *Daphnia pulicaria* (Carotenuto et al., 2005). Additionally, a number of studies have also reported that decadienal reduces the growth of pathogenic and non-marine bacteria (Adolph et al., 2004; Bisignano et al., 2001). Ribalet et al. (2008) showed that PUAs induce different effects on cultured marine bacteria belonging to different taxonomical group, whereas recently two different studies have been conducted on natural bacterial communities exposed to PUAs, reporting different results. On the one hand, PUAs released by diatoms were

found to play an important role in influencing the metabolic activity and composition of natural bacterial communities (Balestra et al., 2011), while, on the contrary, in a mesocosm experiment, high concentrations of PUAs did not influence bacterial or viral abundance and bacterial composition, suggesting that factors other than PUAs are important in determining interactions between diatoms and bacteria (Paul et al., 2012).

A key point about the mode of action of secondary metabolites, including PUAs, is the multiple simultaneous functions that such compounds might have (Ianora et al., 2011a). The evolution of this type of secondary metabolites has been explained in terms of the trend that nature might have had to “catch as many flies with one clap as possible” (Wink and Schimmer, 1999). In terrestrial plants, multiple roles for secondary metabolites are known, and it is believed that this type of action is not a contradiction with respect to their main role for chemical defence and signalling (Wink, 1999).

#### *1.1.3.1 The Effect of PUAs on Diatoms*

PUAs do not affect only copepods, other marine invertebrates and bacteria. Indeed, Casotti et al. (2005) were the first to demonstrate that the PUA decadienal can induce growth inhibition and trigger a mechanism of programmed cell death similar to apoptosis in the marine diatom *Thalassiosira weissflogii*. Such response was found to be time- and dose-dependent, with irreversible effects after 24 h (Casotti et al., 2005). The authors suggested that in marine diatoms PUAs act in a non-toxic manner by signalling changes at the community level when environmental conditions are not optimal for growth, thus representing endogenous controlling factors. These results indicate that PUAs may function not only as chemical defences against grazers, but also as signal molecules to determine cell fate and death within diatom populations. PUAs

have also been shown to act as allelochemicals by inhibiting the growth of different phytoplankton species, thereby possibly conveying an ecological advantage to PUA-producing phytoplankton species compared to non-producing ones (Ribalet et al., 2007a). In particular, PUA production in *S. marinoi* has been reported to increase with ageing of the culture and nutrient limitation, suggesting that there is a link between PUA production, physiological conditions of the cell and nutrient stress (Ribalet et al., 2007b). The main implications of these studies are that during final stages of blooms, when environmental conditions (mainly nutrients) become limiting, stress could act as a signal triggering cell death (Ribalet et al., 2007b). In another study it has been reported that *S. marinoi* produces high PUA levels under silica limitation, suggesting that chemical and mechanical defence are replacing one the other when needed (Ribalet et al., 2009). It has been recently reported that *S. marinoi* cells release PUAs from intact cells immediately before the declining phase of growth, suggesting an infochemical role of PUAs as may occur during blooms (Vidoudez and Pohnert, 2008). In support of the idea of an infochemical role of PUAs in mediating cell-to-cell signalling, it has been demonstrated that the marine diatom *Phaeodactylum tricornutum* can sense the aldehyde decadienal and use it as a signalling molecule to control diatom population size through mechanisms involving programmed cell death (Vardi et al., 2006). In particular, Vardi et al. (2006) found that exposure to decadienal induces a dose-dependent calcium transient leading to nitric oxide (NO) generation via a calcium-dependent nitric oxide synthase-like activity, which ultimately leads to diatom cell death. In this study, cells pretreated with a sublethal dose of PUA, exhibited higher resistance to PUA lethal doses, in comparison to cells that were not pretreated. This suggests that PUAs are sensed by the cells which are thus sensitized and made resistant to following higher concentrations of PUAs (Vardi et al., 2006). It is interesting to note



that also in this stress surveillance system both calcium and NO were found to be involved. Moreover, healthy cells were able to use NO as a signalling molecule to control stressed neighbouring cells (Vardi et al., 2006), and this implies an important role for NO in regulating cell fate of marine phytoplankton (Bowler et al., 2010). In terms of the ecological relevance of this type of pathway in the natural environment in controlling diatom bloom dynamics, a key question is whether a PUA-producing and bloom forming species, such as *S. marinoi*, might have evolved a similar mechanism in sensing and responding to PUA-derived stress.

A subsequent study showed that in *P. tricornutum* the expression of a native gene associated to NO production (named nitric oxide associated protein, PtNOA), increased in DECA-exposed cells (Vardi et al., 2008). *P. tricornutum* cells overexpressing PtNOA exhibited reduced photosynthetic efficiency, reduced growth, altered expression of superoxide dismutase (SOD), enhanced metacaspase expression and increased caspase activity. (Vardi et al., 2008). Both SOD and metacaspase are known to be major players in stress and programmed cell death pathways (PCD) (Bidle and Falkowski, 2004; Wolfe-Simon et al., 2006). The findings related to PtNOA expression demonstrate the importance of cell-death signals both between and within diatom populations. In particular, Vardi et al. (2008) provided evidence that diatom-released chemical cues can be sensed by phytoplankton species, possibly diffusing the message through the population and inducing a cascade of events leading to cell death (Brownlee, 2008). Indeed, a question arises about the possible implications of PUAs in controlling the expression of different death-related genes in diatoms, and in particular, in those diatom species known to produce either PUAs or different secondary metabolites.

The effects of decadienal on benthic diatoms have been investigated in two recent studies (Leflaive and Ten-Hage, 2011a; Leflaive and Ten-Hage, 2011b), reporting that

this PUA was able to inhibit adhesion and motility of the diatom *Fistulifera saprophila*. Also in this case, decadienal was found to induce NO accumulation and, also to inhibit biofilm formation. This latter finding suggests that the presence of PUA-producing diatoms in a biofilm induces selection towards PUA-resistant vs. PUA-sensitive diatom species (Leflaive and Ten-Hage, 2011a). Decadienal was also shown to induce the formation of aggregates in *F. saprophila*, and this response was demonstrated to be species-specific. In another study, the same authors extended their investigation by demonstrating that the effect of decadienal on diatom adhesion was widespread and that it also affected biofilm functioning in terms of photosynthetic efficiency (Leflaive and Ten-Hage, 2011b). Another recent study reported that in the marine microalga *Nannochloropsis oculata* exposure to DECA induced an increase in lipid content (Taylor et al. 2012). The same authors also reported that *S. marinoi* water-soluble extracts induced algal flocculation, suggesting for the presence of a specific infochemicals responsible for the observed event (Taylor et al., 2012). Altogether, these findings point to the hypothesis that PUAs might indeed function as both info and allelochemicals. From a more ecological point of view, a key aspect that remains to be investigated is represented by the physiological responses to PUA exposure in a PUA-producing diatom, (e.g. *S. marinoi*). This would add important insights in the understanding of what types of either protective or stress mechanisms have been developed to exploit PUAs by a PUA-producing species with respect to non-PUA producing ones.

Strain- and clone-specific differences in PUAs and oxyilipin production have been reported in the PUA-producing diatom *S. marinoi*, both on a seasonal and yearly basis (Taylor et al., 2009; Gerecht et al., 2011) In particular, Taylor et al. (2009) suggested that PUA production potentials in *S. marinoi* depends upon the ecological

conditions present at the time of isolation of different strains. Higher production potentials were associated to strains isolated when conditions were less optimal for the survival of the species (Taylor et al., 2009). Additionally, Gerech et al (2011) suggested that such diversity, identified also on a yearly basis, could confer selective advantages to certain clones, consequently shaping diatom population dynamics, and this, in turn, could have important ecological implications during blooming events.

Two recent studies have addressed the key issue of the need of measurement and estimation of PUAs concentrations at sea (Vidoudez et al., 2011a; Vidoudez et al., 2011b). Vidoudez et al. (2011b) performed a survey of the production of PUAs in manipulated mesocosms inoculated with different densities of *S. marinoi*. The authors found that *S. marinoi* was the major source for heptadienal and octadienal during the entire bloom development. Additionally, they reported that particulate decadienal concentrations well correlated with the abundance of the prymensiophyte *Phaeocystis* sp., suggesting that PUA sources other than diatoms should be taken into account when considering the impact of these metabolites. Moreover, in a field study in the Adriatic Sea (Italy), Vidoudez et al. (2011a) reported patchy distribution of PUAs associated with a spring bloom of the diatom *S. marinoi*. The authors reported that *S. marinoi* was the major contributor to the total PUAs. Additionally, they demonstrated that lysis of a diatom bloom can highly contribute to dissolved (i.e. present in seawater) PUA concentrations, and that also other smaller-sized producers have to be considered when estimating total PUA content in the natural environment (Vidoudez et al., 2011a).

In this perspective, it is reasonable to think that, during the course of evolution, natural selection would have likely favoured those phytoplankton species with specific traits that could increase their ecological advantage, for instance avoidance traits (e.g. grazing deterrents) (Smetacek, 2012), but possibly also traits controlling competition

among different phytoplankton species themselves. Unfortunately, this ‘top-down’ aspect of marine plankton ecology has received less attention than the study of those ‘bottom-up’ factors that directly affect phytoplankton growth rates, such as light and nutrient uptake (Smetacek, 2012; Verity and Smetacek, 1996). Ultimately, understanding the different roles that chemical cues and signals play in the marine environment, will be critical to understand the factors that shape the structure and functioning of marine ecosystems (Hay, 2009)

## 1.2 Aims of the Thesis

PUAs function not only as a chemical defence against grazers, but also as signal molecules to determine cell fate and death of diatom populations. They may act both as infochemicals within the same diatom species as well as allelochemicals by inhibiting the growth of closeby species, thereby conferring a competitive advantage to PUA-producing species compared to non-producing ones. The ecological role of these compounds in regulating biological interactions in the natural environment is still under investigation.

My PhD project aimed to investigate the production of nitric oxide (NO) and reactive oxygen species (ROS) in the PUA-producing marine diatom *Skeletonema marinoi* (both NO and ROS) and in the non-PUA producing marine diatom *Phaeodactylum tricornutum* (only NO), as possible stress or signaling molecules in response to PUAs. The objective was to understand whether different response mechanisms to PUAs exist in the two diatom species. Additionally, based on the hypothesis that PUA-producing species might have developed different pathways of resistance to PUAs, I wanted to investigate possible downstream responses at the physiological level in *S. marinoi*, and in particular changes in xanthophylls and carotenoid pigment content and photosynthetic efficiency. Finally, the congeneric diatom *Skeletonema tropicum* was chosen in order to investigate ROS production as well as the expression of death-specific protein genes (*DSPs*) in order to gain new insights in the molecular response to PUAs in marine diatoms.

This thesis is divided into 6 chapters, where the first chapter (i.e. this chapter) is a general introduction presenting the scientific topic of this PhD project and the specific aims of this thesis.

In Chapter 2 NO involvement during optimal growth conditions in the bloom-forming and PUA-producing marine diatom *S. marinoi* is presented. This is based on the observation that previous studies reported high NO levels even in absence of stress factors. The initial work was dedicated to the optimization of protocols for *in vivo* NO detection using fluorescent dye staining and flow cytometry. Additionally, potential involvement of different enzymatic pathways contributing to NO formation in *S. marinoi* has been investigated by the use of different inhibitors. The main finding of this chapter is that in *S. marinoi* NO appears to have an important physiological role in growth regulation. Additionally, for NO production in *S. marinoi*, an enzymatic, nitrate reductase (NR)-dependent pathway appears to be predominant during optimal growth conditions.

In Chapter 3 stress responses to PUAs exposure in terms of NO and ROS production have been studied. In particular the investigation has been carried out in the ecologically relevant diatom species, *S. marinoi*, as compared to the non PUA-producing species, *P. tricornutum*. The question was if PUA-producing species might have evolved different stress response mechanisms with respect to non-PUA producing ones, and if this may underlie their different ecological success in nature. When exposed to DECA, no NO production was observed in *S. marinoi*, contrary to what was previously reported for *P. tricornutum*. Instead, there was a reduced production of NO. A similar response was also observed with OCTA and HEPTA. This was probably due to the consumption of physiological levels of NO, which relate to growth under optimal conditions. In order to exclude a methodological bias, NO production in *P. tricornutum* upon exposure to DECA was tested and confirmed. However, when *P. tricornutum* was exposed to OCTA, the same response of *S. marinoi* was observed, indicating both a species-specific and PUA-specific response. Additionally, *S. marinoi* cells exposed to

the photoinhibitor DCMU presented an increase in NO production, indicating that NO production in *S. marinoi* is likely to be also stress specific. In *S. marinoi*, no increase in ROS was observed upon exposure to DECA, which is not produced by this diatom. Instead, a clear increase was observed upon exposure to the other PUAs tested, OCTA and HEPTA, and with a combination of the two (MIX), suggesting for the presence of a different stress response pathway to PUAs in *S. marinoi*, which appears to be specific for the PUAs produced by the diatom itself. Finally, changes in the protective carotenoid pigments xanthophylls revealed the activation of an antioxidant response in PUAs exposed *S. marinoi* cultures, likely to be activated in order to counteract ROS production. Moreover, no change in photosynthetic efficiency was evident, suggesting that PUAs-exposed cells were able to maintain their photosynthetic capacity and were able to recover after PUA removal.

In Chapter 4 experiments were conducted in order to follow the biochemical pathways of ROS and reactive nitrogen species (RNS) production in *S. marinoi*. A pharmacological approach involving the use of chemical scavengers and enzymatic inhibitors coupled with fluorescent dyes and flow cytometry was applied. The aim was to elucidate the involvement of specific ROS and/or RNS in the stress response of *S. marinoi* to PUA exposure. The main outcome was the identification of a major involvement of the ROS pathway, in particular of superoxide anion ( $O_2^-$ ) and hydrogen peroxide ( $H_2O_2$ ), in the stress response of *S. marinoi* to PUAs.

Chapter 5 presents the results on the expression of two death-specific protein genes (*ScDSP1* and *ScDSP2*) as well as ROS production in response to PUAs in the congeneric diatom *Skeletonema tropicum*. This experimental work was conducted at the National Taiwan Ocean University in Keelung, Taiwan, where *DSPs* in *S. tropicum* were first identified and primers were already available for this study. The goal was to

test the molecular response of genes thought to be involved in a programmed-type of cell death (PCD) and to relate them to ROS production. Indeed, PUAs induced differential *DSPs* expression in *S. tropicum* cells in different growth stages, indicating for a possible involvement of PUAs in controlling PCD at sea. A natural output of this study would be to apply the same approach to *S. marinoi*, once the appropriate molecular tools are identified and optimized.

In the last chapter (Chapter 6) general conclusive remarks are presented. In this chapter, the main outcomes of my PhD project are discussed and presented. Altogether, my data show that a specific pathway of reaction to PUAs is present in diatoms and that this pathway depends on the PUA used. It is proposed that *S. marinoi* has developed specific mechanisms to use OCTA and HEPTA as intra-population infochemical signals, while *P. tricornutum* sees them as allelochemicals. Finally, future perspectives and possible outputs deriving from these results are discussed.



## CHAPTER 2

### Nitric Oxide Production in *Skeletonema marinoi*

#### 2.1 Methodological Aspects

##### 2.1.1 Introduction

Since the development of fluorescent indicators for the detection of endogenous NO *in vivo* (Kojima et al., 1998; Kojima et al., 1999; Itoh et al., 2000), 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) has been extensively and successfully used on different model cells. With excitation/emission maxima of 495/515 nm, DAF-FM-DA can be easily detected by any instrument able to detect fluorescein, including flow cytometers, microscopes and fluorometers, thus making this dye suitable for measurements of endogenous NO in living cells. DAF-FM DA is a membrane permeable ester derivative of DAF-FM. Once inside the cell, the compound DAF-FM DA is first deacetylated by intracellular esterases to become DAF-FM, and then further converted into its fluorescent triazole derivative (DAF-FM T) upon reaction with the NO oxidation product,  $N_2O_3$  (Fernandes et al., 2004). It has been used on cells from a variety of different organisms, including higher plants, marine invertebrates and phytoplankton (Gould et al., 2003; Vardi et al., 2006; Comes et al., 2007; Zottini et al., 2007; Chung et al., 2008; Kim et al., 2008; Thompson et al., 2008; Romano et al., 2011).

The first aim of the laboratory work was to optimize a protocol using flow cytometry to detect NO in *Skeletonema marinoi* using DAF-FM DA. The parameters considered in these tests were:

- optimal dye concentration

- kinetics of staining
- determination of best staining time

In order to avoid false positives, NO donors and scavengers were used (see the following section). Moreover, the presence of NO in *S. marinoi* was confirmed by using the copper-fluorescein complex (CuFL) (Lim et al., 2006b), which is also reported to react with NO in several cell models *in vivo*. CuFL is a relatively novel, cell-permeable fluorescent dye excitable at 488 nm and emitting at 516 nm, in the green (Lim et al., 2006a). CuFL was first synthesized and characterized by Lim et al. (2006b). With respect to the most commonly used diaminofluorescein probes (DAFs), CuFL has been suggested to provide better NO detection in terms of higher specificity for NO, rapidity and brighter fluorescence signals in live cells (Lim et al., 2006b). However, although DAF-FM has been reported to react with NO oxidation products (Kojima et al., 1999), thus making the precise mechanism of intracellular reaction of DAF still complicated to be fully explained, DAF probes are still considered a better choice for the detection of NO production in biological systems due to their favorable properties for cellular imaging applications, their sensitivity to a wide range of NO concentrations and to the fact that their reactivity is independent of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  at physiological concentrations (Gomes et al., 2006, Paul et al., 2011). Given its relative novelty, so far CuFL has been used to detect NO only in murine macrophages and human neuroblastoma cells (Lim et al., 2006b), in human endothelial cells (Efremova et al., 2010) and in the biofilm-forming bacterium *Bacillus subtilis* (Schreiber et al., 2011).

## 2.1.2 Materials and Methods

### 2.1.2.1 Cultures

An axenic culture of the marine diatom *Skeletonema marinoi* Sarno and Zingone, strain CCMP 2092 (formerly *S. costatum*) from the Northern Adriatic Sea, was obtained from the Provasoli-Guillard National Centre for Cultures of Marine Phytoplankton (Boothbay Harbor, USA). This specific strain has been previously thoroughly tested for PUAs production under different growth conditions (Ribalet et al, 2007b; Ribalet et al., 2009). Axenicity was confirmed at the start and the end of each experiment on all the replicates by inoculating 1 mL of culture in 0.1% peptone agar (Sigma Aldrich, Milan, Italy) in seawater medium. In case of contamination samples were discarded and the experiment repeated. Natural seawater, amended with f/2 nutrients (Guillard, 1975) was used as medium. Cultures were maintained in a growth chamber (Hereaeus Holding GmbH, Hanau, Germany) at 23°C on a 12h-12h light-dark cycle under a photon flux density of 110  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  provided by white fluorescent tubes (Phillips TLD 36W/950). Cultures were grown in 2 litres aerated polycarbonate bottles (Fig. II-1). Aeration provided enough mixing to boost growth and to prevent the formation of long chains which would have not allowed the use of flow cytometry. Cell concentrations were monitored by means of both flow cytometry and microscopy. Microscopy counts were obtained from at least 200 cells at 20 x magnification using an Axioskop 2 microscope (Carl Zeiss GmbH, Jena, Germany) and Sedgewick-Rafter counting chambers. For all tests cells were used in their exponential phase of growth at a concentration of  $10^5 \text{ cells mL}^{-1}$  approximately.



Figure II- 1: *S. marinoi* cultures grown in polycarbonate bottles aerated with ambient air for mixing.

### 2.1.2.2 Preparation of Chemicals

The fluorescent NO-sensitive dyes used were: 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) (Molecular Probes, Life Technology, Paisley, USA) and CuFL ("NO-ON" kit by Strem Chemicals, Newburyport, USA). A stock solution of 5 mM DAF-FM DA was prepared in anhydrous dimethylsulfoxide (DMSO) (Sigma Aldrich Inc., Milan, Italy). Aliquots were prepared and stored at -20°C. CuFL was prepared following the manufacturer's instruction; 1 mM FL solutions were prepared in DMSO and aliquots stored at -80°C. A CuFL solution was freshly prepared when needed by adding the copper (II) solution at a ratio of 1:1 at room temperature.

In order to verify DAF-FM DA responsiveness towards detection of NO in *S. marinoi* cells, the NO donor DEA NONOate was used. A 100 mM stock solution of the NO donor DEA NONOate (Vinci Biochem, Vinci, Italy) was prepared in 0.01 M NaOH. The pH of the growth medium was not altered by the addition of NaOH. DEA NONOate concentrations tested were the following: 0.05 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, and 0.5 mM. Although this assay does not specifically test for the probe's specificity towards NO, a NO donor is commonly used to verify the reliability of DAF-FM DA for NO detection in a specific biological system (e.g. Vardi et al., 2006).

The effect of different solvents, which were going to be used for polyunsaturated aldehydes (PUAs) preparation, on DAF-FM DA fluorescence was tested. Solvents tested were absolute methanol (MeOH) (JT Baker, Phillipsburg, NJ, USA), dimethylsulfoxide (DMSO) (Sigma Aldrich, Milan, Italy), and Acetone (Sigma Aldrich, Milan, Italy).

As a negative control, the NO scavenger carboxy-PTIO (cPTIO) (Enzo Life Sciences, Vinci, Italy) was used at a final working concentration of 100 µM, dissolved in filtered sea water (FSW).

The inhibitors used were L-NAME-hydrochloride (LNAME, N<sup>G</sup>-Nitro-L-arginine-methyl ester-HCl) (Enzo Life Sciences, Vinci, Italy) and sodium tungstate dehydrate (ST) (Sigma Aldrich, Milan, Italy). Final working concentrations were 1 mM and 0.5 mM, respectively, dissolved in filtered sea water (FSW).

### 2.1.2.3 Flow Cytometry

Flow cytometry was used to detect NO in live *S. marinoi* cells. The flow cytometer used was a Becton-Dickinson FACScalibur flow cytometer equipped with an air-cooled 488 nm argon-ion laser. The sheath fluid was natural seawater filtered through 0.22 µm polycarbonate filters (Nuclepore, Whatman, Maidstone UK). Optical properties of individual cells were assessed using Forward Angle Light Scatter (FALS) as a proxy of cells size and Right Angle Light Scatter (RALS), which is sensitive to the cell refractive index and internal cell structure, as an indicator of changes in cell internal morphology that are generically termed “granulosity”. Red fluorescence (FL3) was collected through a 650 long-pass filter and was used as a proxy for chlorophyll content. Green fluorescence (FL1) was collected through a 530/30 bandpass filter; both FL1 and FL3 were used in combination with FALS and RALS to identify the population of interest and to detect NO intracellularly as means of green fluorescence. All fluorescence values of samples were normalized to the green fluorescence of the beads used as internal standards (3.7 µm beads Coulter Flow-Set fluorospheres, Beckman-Coulter, Fullerton, USA), and are expressed as units relative to the beads (relative units). Fluorescence values used are represented by the geometric mean of the second peak resulting from DAF-FM DA-derived green fluorescence (see Results, 2.1.3.3). FL3 (red fluorescence) was used as trigger signal with a threshold at channel 52. Data acquisition (10<sup>4</sup> cells on average for each sample), was performed using the CellQuest software (Becton-Dickinson, San José,

USA). Data analysis was performed using both CellQuest software (Becton-Dickinson, San José, USA) and FCS Express 4 Software (De Novo Software, Los Angeles, USA).

### 2.1.3 Results and Discussion

#### *NO detection*

Diethylamine nitric oxide (DEANO) belongs to the NO donor class of NONOates. It generates NO spontaneously with an half life of 2.1 min (Jensen et al., 2000). Tests with DEANO showed a saturation with 0.05 mM of the donor DEANO using the standard protocol (DAF 50  $\mu$ M) (Fig. II-2). The optimal staining time was 20 min with 0.1 mM DEANO. DEANO indeed, confirmed DAF-FM DA specificity for NO detection in *S. marinoi* cells.



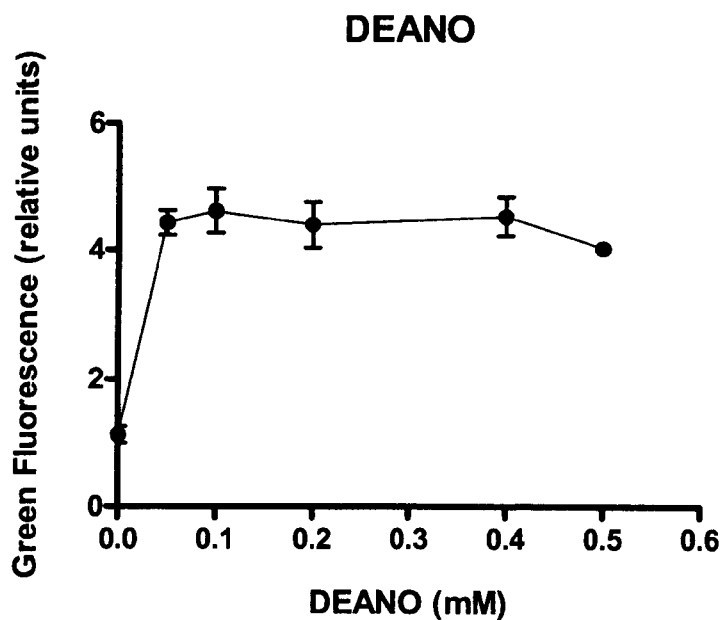


Figure II- 2: *S. marinoi* cells stained with the NO sensitive dye DAF-FM DA in presence of different concentrations of the NO donor DEA-NONOate (DEANO). DAF-FM DA-derived green fluorescence is expressed as units relative to internal standards. Data are means of triplicates  $\pm$  standard deviation (SD).

### 2.1.3.2 DAF-FM DA staining protocol optimization

Once the optimal staining time was found I tested different DAF-FM DA concentrations on *S. marinoi*. An increase in green fluorescence was observed to be linearly correlated with DAF-FM DA concentration. Saturation appeared to occur at 50  $\mu\text{M}$  (Fig. II-3), even though data points do not extend far enough. This concentration was therefore chosen for further use. Since I expected that saturation depends upon the total numbers of cells in the pellet, these were kept constant at ca.  $2 \times 10^5$  cell  $\text{mL}^{-1}$  for a pellet derived from 20 mL of culture, for an estimated total of  $4 \times 10^6$  cells on average in the pellet.

The staining protocol was set as follows:

1. Centrifuge a selected starting volume of the culture at 4300 rpm, at 20 °C for 30'
2. Leave the pellet as dry as possible by gently discarding the supernatant
3. Load cells with DAF-FM DA 50  $\mu\text{M}$ , final concentration
4. Keep samples in the dark by wrapping the tubes in aluminium foil
5. Incubate in the growth chamber for 30' on agitation
6. Dilute with fresh medium (f/2)
7. Spin at 13000 rpm, 20°C for 10'
8. Discard supernatant
9. Re-suspend in fresh medium
10. Dispense in flow cytometry (FCM) tubes
11. Inoculate the samples with DEA NONOate if the case
12. Incubate in the growth chamber for 20'
13. Run FCM samples run up until a total of 10000 events are measured.

In order to exclude an interference of the dark-treatment on inducing DAF-fluorescence therefore generating a false-positive, incubation of samples in light vs. dark was performed. The data show no significant difference between the two treatments (Fig. II-4). As a consequence, the dark treatment was preferred, in order to avoid dye fading.

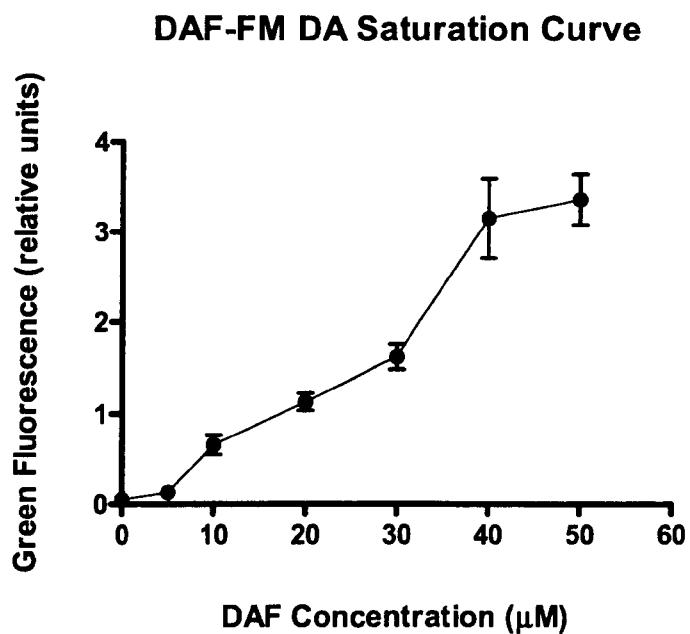


Figure II- 3: *S. marinoi* cells stained with different concentrations of the NO sensitive dye DAF-FM DA. DAF-derived green fluorescence is expressed as units relative to internal standards. Data are means of triplicates  $\pm$  standard deviation (SD).

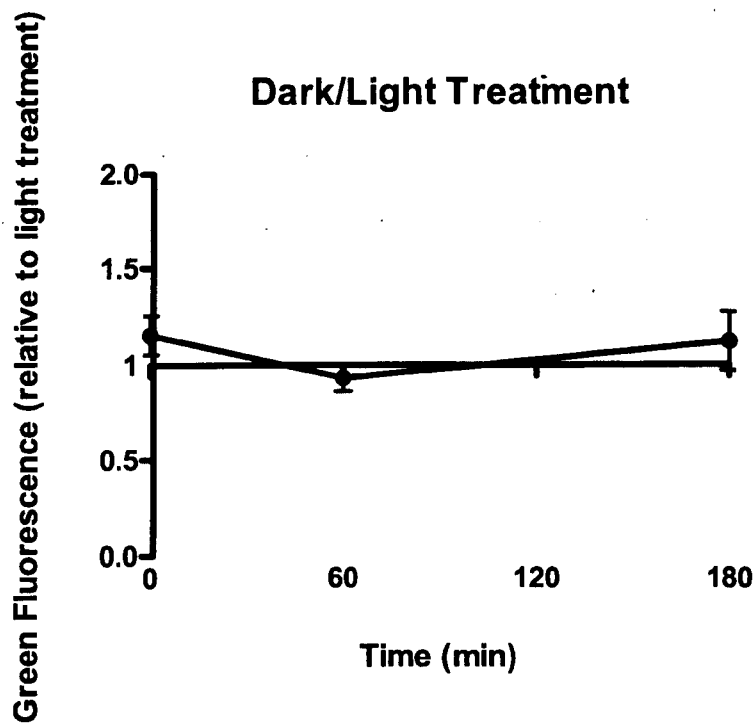


Figure II- 4: *S. marinoi* cells treated with DAF-FM DA. DAF-derived green fluorescence of dark incubated samples relative to DAF-derived green fluorescence of light incubated samples. Data are means of triplicates  $\pm$  standard deviation (SD).

### *2.1.3.3 Patterns of DAF-FM DA green fluorescence*

A recurrent feature in samples stained by DAF-FM DA observed by flow cytometry was a bimodal distribution of green fluorescence in vital cells (i.e. fluorescing red) (Fig. II-5 b-c). The bimodal distribution of green fluorescence was not reflected in any other parameter such as red fluorescence or scatter. The relative proportion of the two peaks was not correlated to DAF concentration or subsequent treatments, except that the NO scavenger cPTIO was having the strongest effect on the cells with higher NO-DAF derived green fluorescence (i.e. on the second peak of DAF-FM-derived green fluorescence) (Fig. II-5 d). cPTIO is a water soluble and stable nitric oxide radical scavenger that shows an antagonistic action against NO both in chemical and biological systems via a radical reaction (Akaike et al., 1993). For these reasons, in the successive analyses, only the specific fluorescence and contribution of this highest peak of DAF-derived green fluorescence was considered for NO detection and thus taken into account.

Other authors have reported heterogeneity with respect to NO production assessed with DAF-FM DA in endothelial cells (Paul et al., 2011), confirming previous findings by Raethel et al. (2003). They demonstrated that this heterogeneity is not due to different phenotypes, but reflects different functional potentiality in NO production, with the lower fluorescence cells not being able to respond using NO to selected stimuli (“non-activated”). Unequal uptake of the dye due to different physiological state of the cells also cannot be excluded.

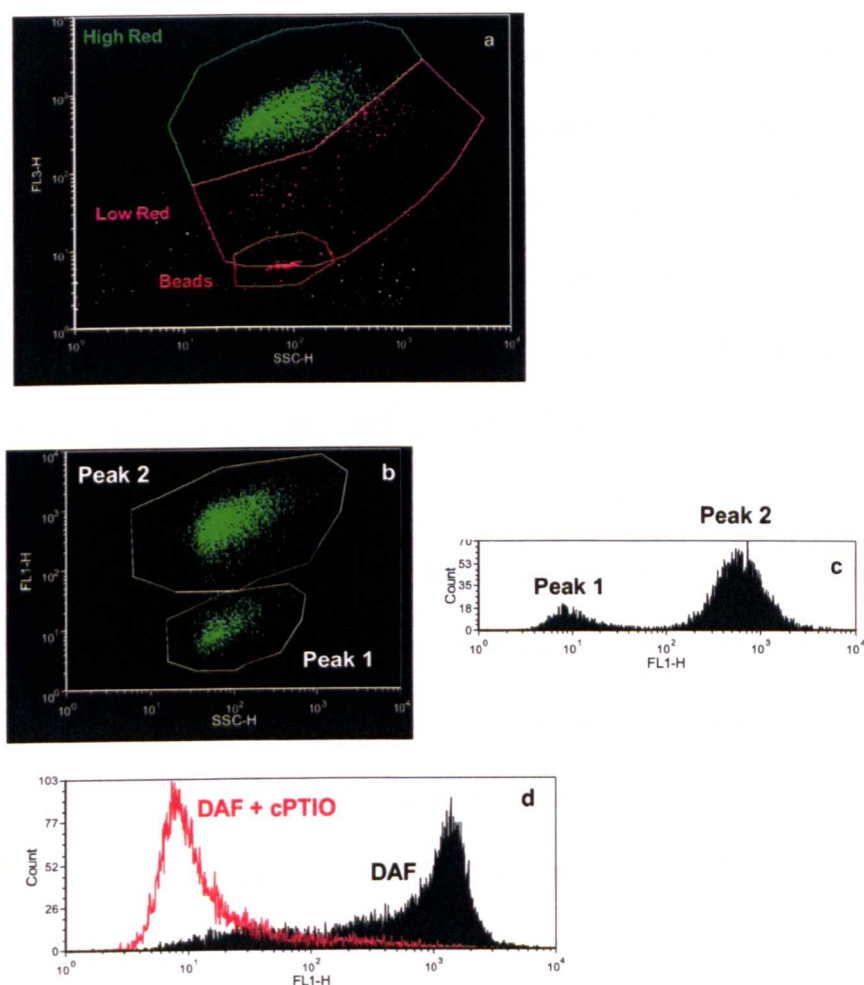


Figure II- 5: (a) Bivariate plot of side scatter (SSC) vs. red fluorescence (FL3) of *S. marinoi* showing one cluster of red-fluorescing cells (b) Dot plot of side scatter (SSC) vs. green fluorescence (FL1) of the “High Red” region indicated in (a); (c) and (d) are histograms showing the distribution of the green fluorescence of cells in region “High Red” of (a); (d) the black profile represents a DAF-loaded sample and the red profile represents a DAF-loaded sample treated with the NO scavenger cPTIO. Beads are fluorescent unimodal particles used as internal standard.

#### 2.1.3.4 Effect of solvents on DAF-FM DA derived fluorescence

Since the dyes used in this study are prepared by dissolving them in different solvents, and also PUAs are dissolved in methanol, I tested the effect of these solvents on the DAF-FM DA-derived fluorescence *in vivo* (i.e. on dye-loaded cells), in order to exclude methodological biases. Solvents tested were MeOH, DMSO and Acetone at different concentrations, namely  $1\ \mu\text{L mL}^{-1}$ ,  $2.5\ \mu\text{L mL}^{-1}$  and  $5\ \mu\text{L mL}^{-1}$  for MeOH and  $0.01\ \mu\text{L mL}^{-1}$ ,  $0.1\ \mu\text{L mL}^{-1}$ ,  $0.5\ \mu\text{L mL}^{-1}$ ,  $1\ \mu\text{L mL}^{-1}$  and  $2.5\ \mu\text{L mL}^{-1}$ , for both DMSO and Acetone.

MeOH was influencing the least the DAF-NO fluorescence with respect to the control (i.e. DAF-stained samples with no solvent addition) (Fig. II-6 a). This shows that the solvent was not having any effect in either quenching or increasing the NO-DAF-derived green fluorescence. Instead, DMSO or Acetone induced an initial increase in green fluorescence (at  $T_0$ ), with a subsequent decrease after 30 min, relative to the control (Fig. II-6 b and c, respectively). Acetone-induced fluorescence was 0.5 higher than the control at  $T_0$  and 0.5 lower than the control afterwards (Fig. II-6 c). The subsequent decrease was much lower for the DMSO (0.24 on average) (Fig. II.6 b). Therefore, MeOH was chosen as the most suitable solvent to be used for PUAs preparation. The use of the NO scavenger cPTIO confirmed a reduced NO-DAF derived green fluorescence (data not shown).



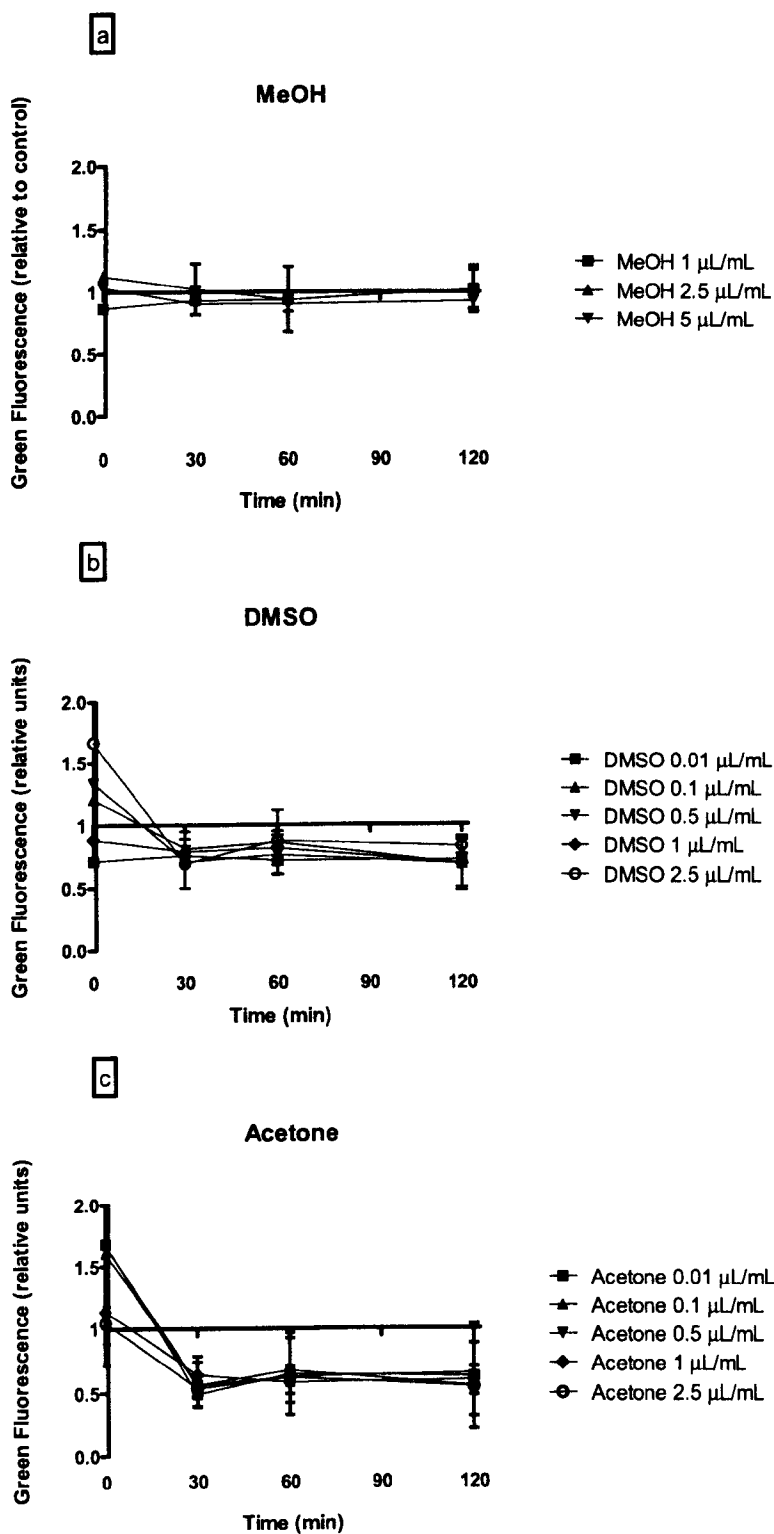


Figure II- 6: Effects of three different solvents on DAF-FM DA derived green fluorescence; (a) MeOH; (b) DMSO; (c) Acetone. Data are means of triplicates  $\pm$  SD.

#### 2.1.3.5 CuFL staining

The fluorescent NO-sensitive dye CuFL was also tested to confirm NO detection in *S. marinoi* cells. No specific staining protocol was available for algal cells. However, other users have used the same protocol as for DAF-FM DA with higher plant leaves (Delledonne and Vandelle, pers.comm.). In the case of my biological system, the protocol was not suitable, as the CuFL-derived green fluorescence was too weak to be detected. This was likely to be dependent on dye removal due to washing steps required for the DAF staining protocol. Therefore, tests were performed to find the optimal procedures, involving reduction of washing steps and different dye concentrations.

Saturation was reached at 10  $\mu$ M, and this concentration was set as the optimal to be used in further experiments (Fig. II-7). As for duration of the incubation time, binding of the dye was immediate, as the maximal fluorescent was measured very shortly after staining (Fig. II-7).

In summary the CuFL protocol was much simpler, requiring only little manipulation and loading time with respect to DAF-FM DA. However, based on its more difficult availability, higher price and uncommon use in NO-related studies (being a relatively new dye), DAF-FM DA was still preferred for routine use.

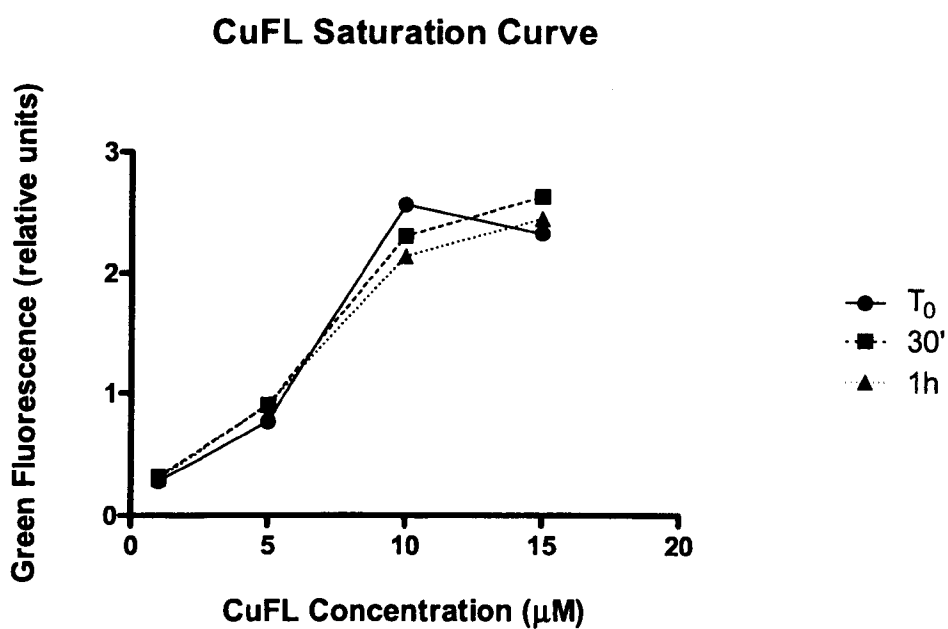


Figure II- 7: *S. marinoi* cells stained with different concentrations of the fluorescent NO sensitive dye CuFL. CuFL-derived green fluorescence is expressed as units relative to internal standards. Data are means of triplicates  $\pm$  standard deviation (SD). The legend on the right represents the different incubation times tested.

## **2.2 NO Production in *S. marinoi* during Optimal Growth Conditions**

### **2.2.1 Introduction**

#### *2.2.1.1 Nitric Oxide in Biological Systems*

The history of NO research started in the early 1990's, after the first identification of NO as an endothelium-derived relaxing factor in rabbit aortic endothelial cells (Ignarro et al., 1987; Palmer et al., 1987). The great majority of studies that initially addressed the functions and roles of NO in biological systems focused on animal cells (Nathan and Xie, 1994; Schmidt and Walter, 1994). However, it also became soon evident that the use of NO is not exclusive of the animal kingdom (Beligni and Lamattina, 1999a; Durner and Klessig, 1999; Gouvea et al., 1997). In fact, NO has been reported to be a multipurpose molecule in animals (Wink and Mitchell, 1998), as well as plants (Lamattina et al., 2003), fungi (Carmona et al., 2012) and bacteria (Plate and Marletta, 2012). In particular, it is now well established that NO is a key molecule in many different physiological and defence processes in plants. In particular NO has been found to regulate stomatal closure in guard cells (Neill et al., 2002a), to stimulate germination (Beligni and Lamattina, 2000) and growth (Pagnussat et al., 2003), to inhibit flowering (He et al., 2004) and also to function as a signal molecule during disease resistance (Delledonne et al., 1998). Moreover, NO also plays a role in the regulation of a number of different genes, involved for instance in pathogen responses, photosynthesis, cell death and basic cellular metabolism (for a review see Wendehenne et al., 2004).

In animals, NO is produced enzymatically by nitric oxide synthase(s) (NOS) which are a group of isoenzymes responsible for the conversion of L-arginine to L-citrulline with the concomitant production of NO and reduction of NADPH to NADP<sup>+</sup>. Three isoforms of NOS have been identified, based on the tissues from which they were originally

isolated: neuronal NOS (nNOS), inducible NOS (iNOS in macrophages) and endothelial NOS (eNOS) (Nathan and Xie, 1994). nNOS and eNOS genes are constitutively expressed, whereas the iNOS gene is expressed in macrophages and other cell types only during inflammatory conditions and in response to cytokines (Mayer and Hemmens, 1997). In plants, the actual NO biosynthetic pathway has been long debated. Two main enzymatic pathways are recognized to be responsible for NO biosynthesis in plants, a NOS-like pathway and a nitrate reductase (NR) pathway (Gas et al., 2009). Yamasaki et al. (1999) provided evidence for the first reported mechanism involved in NO production in plants. These authors found that NO production was dependent on the reduction of nitrite to NO, catalyzed by the enzyme nitrate reductase (NR), an enzyme present in the cytosol that reduces nitrate to nitrite. However, the contribution of NR to NO synthesis in plants is still considered controversial (Gas et al., 2009). In terms of NOS-dependent NO synthesis in higher plants, two main sources of evidence were reported. On one hand, initial work demonstrated the production of NO and L-citrulline from L-Arg by plants extracts and its inhibition by specific inhibitors (Delledonne et al., 1998; Durner et al., 1998). On the other hand, a different approach employed was based on the use of antibodies against mammalian NOS enzymes (Barroso et al., 1999; Ribeiro et al., 1999). However, the lack of NOS genes homologs of the mammalian ones within the published *Arabidopsis* (Arabidopsis Genome, 2000) and rice (Yu et al., 2002) genome sequences, have started to question the existence of a NOS-like enzyme in plants. In recent years there have been two papers reporting the identification of a NOS gene in higher plants (Chandok et al., 2003; Guo et al., 2003). However, findings reported in both works were subsequently demonstrated to be non reproducible. In particular, Chandok et al. (2003) reported a NOS activity for a variant of the P protein of the glycine decarboxylase complex; this finding was later retracted since the authors were unable to confirm their

previous results (Travis, 2004). Guo et al. (2003) identified the second potential NOS gene in *Arabidopsis thaliana*, AtNOS1, based on sequence similarity to a protein that has been implicated in NO synthesis in the snail *Helix pomata*. However, since it was not possible to reproduce the reported results, the validity of this finding was later questioned (Crawford et al., 2006; Zemojtel et al., 2006). In particular, Moreau et al. (2008) finally reported that the questioned gene was a cGTPase and not a NOS gene. A major breakthrough came recently, when Foresi et al. (2010) reported the identification of a NOS enzyme in the plant kingdom, and in particular from *Ostreococcus tauri*, a unicellular green alga. This organism is particularly relevant since it belongs to the Prasinophyceae (Chlorophyta), an ancestral class within the green plant lineage that originated through the primary endosymbiosis from which photosynthetic eukaryotes evolved (Falkowski et al., 2004). This study, in turn, supports the possibility of the existence of a NOS enzyme in plants (Eckardt, 2010).

What is nowadays generally accepted is that there are likely to be different potential biosynthetic pathways for NO production in plants. First, NO could be produced through a NOS-like enzyme, given the fact that a number of studies have reported a NOS-like activity in plants (e.g. Del Rio et al., 2004). Second, another source for NO is dependent on the activity of the enzymes Nitrate Reductase (NR) and Nitrite Reductase (NiR). The primary function of NR is related to nitrate ( $\text{NO}_3^-$ ) uptake, but it can also generate NO from nitrite ( $\text{NO}_2^-$ ), even though the reduction efficiency has been estimated to be low (~1% of NR activity) (Rockel et al., 2002). Additionally, other enzymes have been reported to be responsible for NO generation in plants. (Stohr and Stremlau, 2006) found that a plasma-membrane-bound enzyme, nitrite-NO-oxidoreductase (Ni-NOR) is active in plant roots, whereas nitrite has been shown to be reduced to NO by mitochondria in tobacco (Planchet et al., 2005) and by chloroplasts in soy bean (Jasid et al., 2006), in which it was

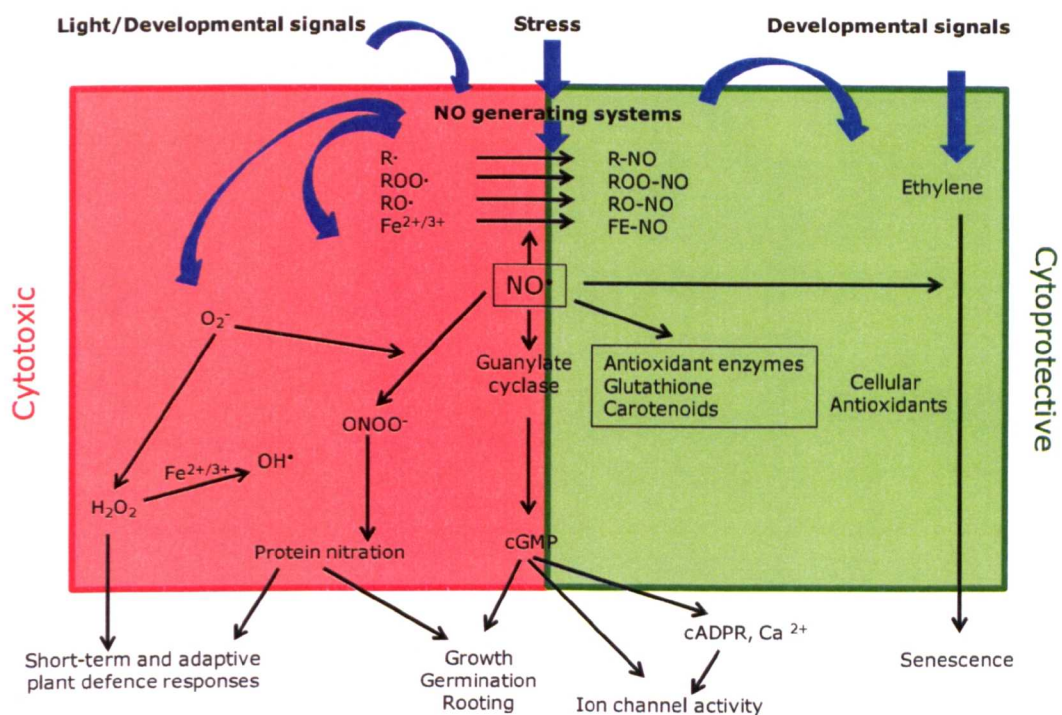
found that also arginine can be used as a substrate. Finally, a non enzymatic NO biosynthetic pathway under acidic conditions has also been suggested to exist in plant tissues (Bethke et al., 2004).

From a cellular point of view, still little is known about the transduction processes that lead to such a wide range of effects reported to occur in response to NO production. The main signalling processes occurring downstream of NO production include either direct or indirect types of responses (Moreau et al., 2010). Direct mediation involves post translational modification of proteins, mainly by reversible S-nitrosylation of cystein residues and irreversible tyrosine nitration (Moreau et al., 2010). On the other hand, three main forms of secondary messengers are thought to be involved in the indirect mediation of NO signalling: cyclic guanosine monophosphate (cGMP), cyclic ADP ribose (cADPR) and protein kinases induced by cytosolic  $\text{Ca}^{2+}$  (Moreau et al., 2010; Wendehenne et al., 2004; Wilson et al., 2008; Besson-Bard et al., 2008). It is however important to consider that NO can exist in different redox forms, and each might elicit different cellular responses. More specifically NO can exist as three different interchangeable species: the radical NO ( $\text{NO}^\bullet$ ), the nitrosonium cation ( $\text{NO}^+$ ) and the nitroxyl radical ( $\text{NO}^-$ ). Being a reactive free radical, the half-life of NO is relatively short, in the order of a few seconds, so the range of the different effects it can cause is confined to the cellular environment where it is produced or to cells in close proximity (Neill et al., 2003).

In a broader picture, when considering different free radical species, other reactive compounds also play an important role in plant signalling responses. The best known and studied reactive compounds are the so-called Reactive Oxygen Species (ROS), which include superoxide anion ( $\text{O}_2^-$ ), hydroxyl radical ( $^\bullet\text{OH}$ ) and non-radical active species such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). In terms of interaction between NO and ROS there seems to be a contradiction between a potential role of NO in neutralizing the negative

effect of ROS versus a synergistic deleterious effect of both NO and ROS (Beligni and Lamattina, 2001). However, it is important to consider that such a contradiction might not actually exist, since for NO itself it has already been well established that it can have both cytotoxic and cytoprotective roles at the cellular level, depending on its concentration (Lamattina et al., 2003). Based on the review of Beligni and Lamattina (2001), an attempt to summarize this dual role of NO is presented in Fig. II-8.





Adapted from Beligni and Lamattina (2001)

Figure II- 8: Schematic representation of the potential effects of NO in plants.  $RO^\bullet$  = non oxygen free radicals;  $R^\bullet$  = alcoxyl radicals;  $ROO^\bullet$  = peroxy radicals;  $OH^\bullet$  = hydroxyl radical;  $ONOO^-$  = peroxynitrite

The key point is that depending on the cell state, NO can lead either to cell survival or cell death as a response to external conditions. The cytoprotection is mainly related to the involvement of NO in the regulation of the level and toxicity of ROS (Halliwell and Gutteridge, 1984). NO redox chemistry is hypothesized to provide a general mechanism for cell redox homeostasis regulation (Stamler et al., 1992). As a consequence, NO can have a protective action against oxidative stress caused by increasing levels of superoxide, hydrogen peroxide and alkyl peroxides (Wink et al., 1995). The Fenton-type reaction between  $H_2O_2$  and redox active metal produces the hydroxyl radical ( $\cdot OH$ ). This is a powerful oxidant which can oxidize different biomolecules causing deleterious cellular effects (Ames et al., 1993). NO can attenuate the oxidative damage derived by this reaction by scavenging iron or superoxide thus limiting hydroxyl radical formation (Beligni and Lamattina, 2001; Beligni and Lamattina, 2002; Wink et al., 1995). NO has also been reported to prevent lipid peroxidation by acting as a chain-breaking antioxidant (Hogg et al., 1993; Rubbo et al., 1994). The cytoprotective effects of NO in plants have been reported under both biotic and abiotic stress factors, for instance during infection of the pathogen *Phytophthora infestas* in potato leaves or under strong photo-oxidative conditions (Beligni and Lamattina, 1999a; Beligni and Lamattina, 2002). Cytoprotection against oxidative stress has also been shown to be active at different levels of organization (e.g. tissue, organ and whole plant), as well as to be directed towards different biomolecules, such as DNA, RNA, protein and lipids (Beligni and Lamattina, 1999a; Beligni and Lamattina, 1999b; Beligni and Lamattina, 2002). Indirect pathways of protection could also derive from the interaction or activation by NO of the cellular antioxidant system (e.g. glutathione, antioxidant enzymes, etc.) (Bavita et al., 2012; Krasuska and Gniazdowska, 2012). On the other hand NO-mediated cytotoxicity is generated mainly by reaction with superoxide anion ( $O_2^-$ ), leading to the formation of the

powerful oxidant peroxynitrite ( $\text{ONOO}^-$ ) (Huie and Padmaja, 1993), which has been shown to be deleterious to lipids, proteins and DNA (Lipton et al., 1993). Moreover, all these different responses might involve either secondary messengers (e.g. cGMP, cADPR and  $\text{Ca}^{2+}$ ), or the direct modification of target molecules (e.g. through S-nitrosylation) (Moreau et al., 2010).

Additionally, an interplay between light or developmental factors and NO signalling could have an important role also in regulating processes such as growth, flowering, germination and senescence (Beligni and Lamattina, 2000; Leshem and Pinchasov, 2000). In particular, light-driven germination by NO has been reported for the higher plant *Paulownia tormentosa* (Grubisic and Konjevic, 1990) and for lettuce seeds (Beligni and Lamattina, 2000). On the other hand, evidence of the interconnection between NO and ethylene in the maturation and senescence of plant tissues suggests an antagonistic effect of these two molecules during these developmental stages (Leshem et al., 1998; Leshem and Pinchasov, 2000). This could imply that changes in NO levels determines the alteration of some important signals involved in ethylene-mediated developmental processes (Lamattina et al., 2003; Schaller, 2012).

Considering that many diverse NO-related physiological and developmental responses to NO have been reported to occur in plants, it seems reasonable to assume that changes in the gene expression profile do indeed occur following NO production (Neill et al., 2003; Wendehenne et al., 2004). NO induction of gene expression was first reported during plant-pathogen interactions in tobacco (Durner et al., 1998). Additionally, the NO dependent pathogenesis-related (*PR*) gene expression in tobacco mosaic virus (TMV) infection was reported (Klessig et al., 2000). Delledonne et al. (1998) showed that a NO donor could induce both phenylalanine ammonia-lyase (*PAL*) and chalcone synthase (*CHS*) gene expression in soybean suspension cultures. Subsequently, UV-B induction of

*CHS* in *Arabidopsis* has also been demonstrated (A.-H.-Mackerness et al., 2001). In *Arabidopsis*, NO was also found to induce ferritin mRNA accumulation, and to be necessary for iron induction of ferritin transcript accumulation (Murgia et al., 2002). In a microarray study conducted on *Arabidopsis*, a large number of NO-induced genes were identified (Huang et al., 2002). In particular, alternative oxidase genes, pathogenesis related genes, antioxidant genes including peroxidases and glutathione-S-transferases, as well as some genes thought to encode signalling proteins (Huang et al., 2002). Later, a NO-dependent activation of genes involved in disease resistance, photosynthesis, cellular transport and programmed cell death has also been reported (Polverari et al., 2003). In terms of the mechanism by which NO is thought to induce gene expression, it is hypothesized that NO could either alter transcriptional profiles directly, e.g. NO can diffuse into the nucleus and activate or inactivate transcription factors in several ways, including S-nitrosylation and interaction with transition metals (Neill et al., 2003), or it could indirectly modulate transcription factors activity in signalling cascades, e.g. NO induction of *PR1* gene expression in tobacco through salicylic acid (SA) synthesis and action (Durner et al., 1998).

It is likely that depending on NO concentration, timing of production and cellular localization, different biological responses will occur under a given circumstance (i.e. protective, regulatory and detrimental effects) (Wink and Mitchell, 1998). From a general point of view the biological effects of NO can be direct or indirect. The former include those direct interactions between NO and biological molecules, and normally occur at low NO concentrations. These direct effects are also defined as those whose rates are fast enough to be considered physiologically relevant. In particular, direct effects of NO are directed towards metal complexes, metal-oxygen complexes and lipid radicals (Wink and Mitchell, 1998). Indirect effects are, instead, dependent on the interaction between NO

with either superoxide anion or oxygen, which gives rise to reactive nitrogen (oxide) species (RNOS), which can subsequently give rise to oxidation, nitrosation and nitration processes (Wink and Mitchell, 1998; Ischiropoulos et al., 1992; Beckman et al., 1992).

#### 2.2.1.2 Nitric Oxide in Marine Phytoplankton

Compared to the growing evidence for a role of NO in both animals and plants, NO production and its effects in marine phytoplankton have received little attention until relatively recently. The first report of NO in the chlorophyta *Scenedesmus obliquus* was by Mohn and Soeder, (1996). Subsequently, it was reported that NO modulated phototaxis in *Chlamydomonas reinhardtii* (Lobysheva et al., 1996). NO involvement in the symbiotic relationship between dinoflagellate algae and anthozoans was also first reported (Trapido-Rosenthal et al., 1996), and a role for NO and NOS activity as ecotoxicological biomarkers in marine tropical environmental was suggested (Morrall et al., 1998). Later, Mallick et al. (1999) found NO production to occur also in the nitrogen-fixing cyanobacterium *Anabena doliolum*, suggesting that NO production could be a common feature of algae. In addition they found a nitrite ( $\text{NO}_2^-$ ) dependent production of NO and the critical role of the enzyme NR in providing the substrate ( $\text{NO}_2^-$ ) for NO release. Since these early studies, the actual pathway of NO production in marine algae has been a major topic of debate. A number of studies conducted on microalgae also confirmed that nitrate reductase plays a key role in NO production (e.g. Sakihama et al., 2002), whereas other authors reported a NR-independent NO production (Tischner et al., 2004). Others provided evidence that a NOS-like activity mediates NO production in marine phytoplankton, thereby suggesting a pathway dependent on the conversion of L-arginine into L-citrulline (Kim et al., 2006). Further work conducted on the chlorophycean *Scenedesmus obliquus* reported that NO plays a protective role against the negative

effects produced by the reactive oxygen species hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) on chlorophyll *a* and growth yield (Mallick et al., 2002). Results from a different work revealed that NO synthesis was enhanced upon exposure to different types of toxic chemicals (e.g. herbicides) and was strongly dependent on light intensity, indicating a close integration of photosynthesis and nitrogen metabolism due to nitrite availability through the involvement of the enzyme NR (Mallick et al., 2000a). Under standard growth conditions the main role of NR is to catalyze the reduction of nitrate to nitrite, which is subsequently reduced to ammonium by the enzyme nitrite reductase (NiR) localized within the chloroplasts of microalgae (Bouchard and Yamasaki, 2008). Nitrite reduction requires reducing equivalents produced by the photosynthetic electron transport chain (Crawford, 1995). Under stress conditions, which can result in the impairment of the photosynthetic apparatus, nitrite can accumulate inside the cytoplasm and be converted by NR to NO through NR-dependent enzymatic activity in *S. obliquus* (Yamasaki and Sakihama, 2000). A NR-dependent NO production has also been reported in the green alga *Chlamydomonas reinhardtii* (Sakihama et al., 2002). Another pathway identified in the unicellular green alga *Chlorella sorokiniana* as a possible mechanism involved in NO generation is the mitochondrial electron transport (Tischner et al., 2004). Using NR-deficient mutants the authors found that under anoxic conditions NR was not the only source of NO, but rather mitochondrial electron transport was responsible for the reduction of nitrite to NO through the electron flow from complex III to complex IV via cytochrome *bc*, suggesting that mitochondria can produce NO under anoxia on sufficient nitrite supply.

In the marine chlorophyta *Dasycladus vermicularis* NO was found to be involved in wound-responses together with hydrogen peroxide  $\text{H}_2\text{O}_2$  (Ross et al., 2006). Moreover, the respective activation of these reactive molecules was found to be partly co-regulated,

supporting the evidence that a signalling relationship exists between reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Neill et al., 2002b; Planchet et al., 2005). Additionally, Ross and co-workers (2006) found that in *D. vermicularis* NO production is dependent on NR and not on a NOS-like enzyme. However, it should be noted that NO generation in an Antarctic *Chlorella* sp. was correlated to an increased activity of both NR and NOS-like enzymes, indicating that they could both be potential contributors to the cellular production of NO (Estevez and Puntarulo, 2005). The authors speculated that NR and NOS-like activities could be dependent on the cells growth phase. Indeed, it should be mentioned that, other authors suggested that in photosynthetic organisms NO is produced both as a 'primary product' via an active route through a NOS-like enzyme, or as a 'byproduct' through NR acting as a cytotoxic molecule leading to impaired cellular metabolism (Takahashi and Yamasaki, 2002; Sakihama et al., 2002). In the first case NO would be stoichiometrically synthesized and highly regulated, and act as a messenger for signal transduction pathways (Sakihama et al., 2002).

Kim et al. (2006) observed that the raphidophyte *Chattonella marina* was able to produce NO under optimal growth conditions and that such production was dependent on the activity of a NO synthase-like enzyme. Later on it was shown that NO production also occurs in other species of raphidophytes, i.e. *Chattonella ovata* and *Heterosigma akashiwo*, known to be highly ichthyotoxic, suggesting that NO might be involved in a detrimental effect on fish gills. NO has also been related to coral bleaching. In fact in the dinoflagellate *Symbiodinium bermudense* NOS-like activity was observed only in cells freshly isolated from their cnidarians host, and not from the microalgae grown in culture, possibly indicating that NO is needed in the symbiotic interaction (Trapido-Rosenthal et al., 2001). On the other hand, Perez and Weis (2006) report NO production only in the coral host and not in the dinoflagellate. Bouchard and Yamasaki (2008, 2009) have also

investigated NO involvement in the symbiotic relationship in corals. In this case, *Symbiodinium microadriaticum* displayed a temperature-dependent NO production both via a NR and a NOS-like pathway. In particular, NO production by *S. microadriaticum* was found to increase at high temperatures (Bouchard and Yamasaki, 2008), suggesting that heat stress induces NO generation. This is particularly relevant in the context of global warming and its role in coral bleaching. Subsequently, the same authors found a strong correlation between NO and the increase in caspase 3-like activity leading to cell death, suggesting a role for NO in programmed cell death (PCD) induction in the coral symbiotic microalgae (Bouchard and Yamasaki 2009).

In the marine diatom *S. costatum* NO has been reported to be produced physiologically under optimal conditions (Zhang et al. 2003; Zhang et al., 2006b). The effect of NO appears to be related to its intracellular concentrations. When present at low concentrations it is correlated to increased growth rate, while at high concentrations it is toxic to cells. This suggests a role as growth regulator (Zhang et al., 2003). NO is indeed related to growth phases in different phyla of marine phytoplankton, including diatoms (Zhang et al., 2006b). In this work the authors found that under optimal growth conditions in culture two major peaks of NO occurred, one two or three days before the maximal cell density during the exponential phase, and a second (smaller) peak right before the decay phase, similar to what has been observed in higher plants (Pedroso et al., 2000). Zhang et al. (2006a) also reported that NO production is modulated by environmental factors including light, temperature, salinity and trace metals.



## 2.2.2 Results

### 2.2.2.1 NO basal levels

To detect NO basal levels cultures were sampled daily until the stationary phase was reached (except day 5). The time of sampling and measurement of NO content was kept constant every day. Cultures were harvested and incubated with DAF-FM DA according to the previously described protocol (see section 2.1.3.2) and then run for flow cytometry to measure NO endogenous levels. Indeed, the observed decrease in NO levels was directly related to NO cell content rather than to dye fading. The average growth rate of *S. marinoi* during the exponential phase under optimal growth conditions was  $0.74 \text{ d}^{-1} \pm 0.25 \text{ d}^{-1}$ . The average maximal cell concentration at stationary phase was  $7.15 \cdot 10^5 \text{ cell mL}^{-1} \pm 7.15 \cdot 10^4$  (Fig. II-9).

After time zero (i.e. time of inoculation), NO levels showed a lag phase of 3 days with  $1.34 \pm 0.34 \text{ r.u.}$ , corresponding to the lag phase of growth of the cultures (Fig. II-9). Values then increased with the exponential phase of growth until the beginning of the stationary phase ( $10.84 \pm 0.18 \text{ r.u.}$ ). Indeed, the reduction in NO levels seemed to occur when cell numbers were still increasing (at day 9), although not exponentially, and right before cultures reached the stationary phase (at day 11). Then green fluorescence stopped to increase again until the beginning of the decay phase of the culture (at day 19) (Fig. II-9).

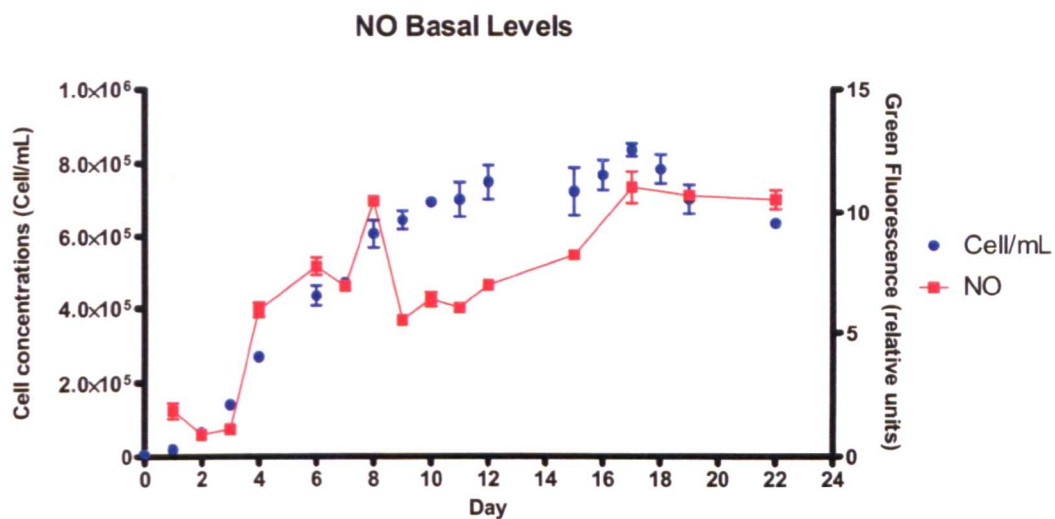


Figure II- 9: Average concentrations of *S. marinoi* during growth in bubblings in standard growth conditions (see Material and Methods 2.1.2.1). Data are means of triplicates  $\pm$  standard deviation (SD). NO is expressed as green fluorescence relative to internal standards.

#### 2.2.2.2 Use of NOS and NR inhibitors

In order to obtain indications on the involvement of either one of the two known enzymes implicated in NO synthesis in phytoplankton, NR and NOS, L-NAME, a nitric oxide synthase (NOS) inhibitor, and sodium tungstate (ST), a nitrate reductase inhibitor, were tested on exponential phase cultures. LNAME ( $N^G$ -Nitro-L-arginine-methyl ester . HCl) has been reported to be a general inhibitor of nitric oxide synthase (NOS) in mammalian cells (Rees et al., 1990), while it is known that tungstate can be substituted for molybdenum (Mo) and inhibit NR activity by preventing the formation of an active molybdenum cofactor, that is necessary for the catalytic activity of NR (Notton and Hewitt, 1971). Even though tungstate specificity for NR inhibition has been recently suggested to be treated with caution (Xiong et al., 2012), thanks to its simple application and common availability, tungstate has been widely used as a NR inhibitor in plant and algal NO research (Mallick et al., 1999; Freschi et al., 2010; Negi et al., 2010).

The selected incubation times for inhibitors were chosen in order to allow a greater inhibition of NO enzymatic biosynthesis (Pfeiffer et al., 1996). L-NAME had no effect in inhibiting NO production while ST did, and in a comparable way of the NO scavenger cPTIO, suggesting that NR was involved. The highest inhibition was obtained after 210 min, with a decrease of  $35.88 \% \pm 2.32 \%$ , comparable to  $46.43 \% \pm 6.83 \%$  decrease of DAF-positive cells with the NO scavenger cPTIO (Fig. II-10).

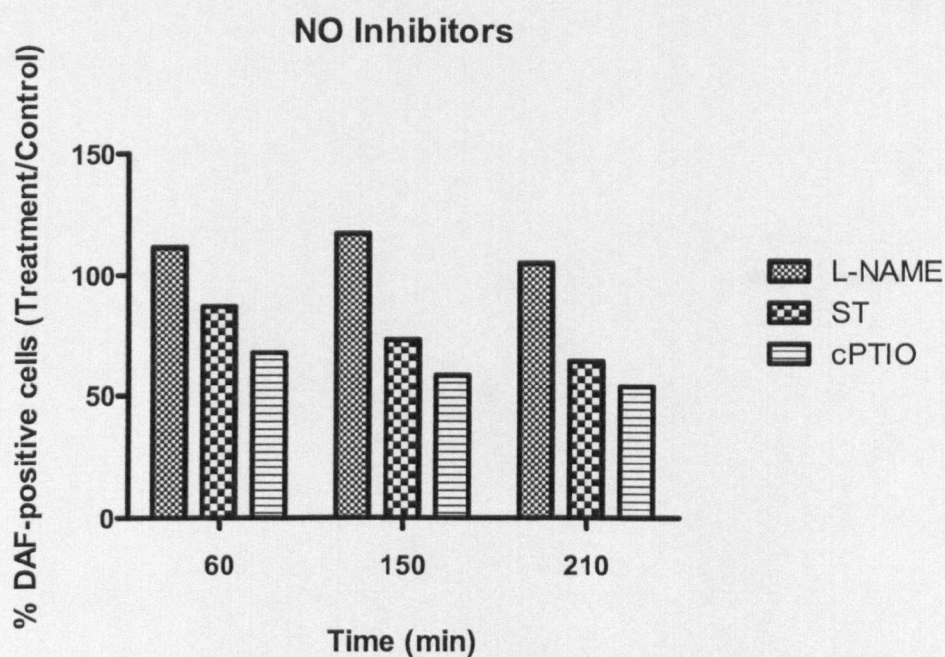


Figure II- 10: % of DAF positive cells with respect to the control (*S. marinoi* cells loaded with DAF and not treated with any inhibitors) when two inhibitors of two different enzymes involved in NO production are used. LNAME ( $N^G$ -Nitro-L-arginine-methyl ester-HCl); ST (sodium tungstate). cPTIO (carboxy-PTIO) is a NO scavenger and represents the negative control. Data are means of duplicates from two independent cultures.

### 2.2.3 Discussion

NO production in *S. marinoi* during optimal growth appeared to vary with growth phases. The highest NO production was indeed coincident with two key passages; from exponential to stationary and from stationary to decay, suggesting a physiological role of NO in these two critical steps of the life of a culture.

Zhang et al. (2006a) also found similar patterns in three different marine microalgae, including *Skeletonema costatum*, with an increase in NO two to three days before the maximal cell density. A second peak was also reported to occur during the declining phase of growth. As in the case of my experiments, the observed NO variability suggests a role of NO as a growth-related factor, as also reported for higher plants (Leshem, 1996; Pagnussat et al., 2002; Sirova et al., 2011; Gouvea et al., 1997). Moreover, Zhang et al. (2006a) reported a correlation between NO and nutrient consumption as well as pH in the culture medium, suggesting that NO could function as an indicator of algal growth status. Additionally, different factors that can influence algal growth, such as light, temperature, salinity and trace metal concentrations, were also found to affect NO concentration (Zhang et al., 2006b). This implies that the specific conditions of algal growth are strongly reflected in NO production, and this should be taken into account when measuring NO dynamics and its modulation. In freshwater antarctic *Chlorella* sp., NO increases at the beginning of the log phase of growth, suggesting that NO may act as a trigger or an indicator of the passage to active growth (Estevez and Puntarulo, 2005). Interestingly, the same authors pointed out that such a response was not observed in *Chlorella* cells from temperate regions, providing further evidence that NO is a signalling molecule strongly modulated by growth conditions. Additionally, in the freshwater, bloom-forming cyanobacterium *Microcystis aeruginosa*, a strong linear relationship between cell density and NO concentration was found (Tang et al., 2011). The same

authors also suggested that a correlation exists between NO increase and the increase in both cell density and Chl-a content, up to values reported for cyanobacterial blooms in the field. This points to a role of NO as an indicator for *M. aeruginosa* growth status and its potential role as predictor of the occurrence of a bloom. A possible involvement of NO in the stimulation of red tides in the natural environment has also been suggested by Zhang et al. (2005). NO production during standard growth conditions has also been reported in the HAB species *Chattonella marina* (Kim et al., 2006) and *Chaetoceros curvisetus* (Zhang et al., 2006b). Interestingly, Kim and co-workers (2008) reported that significant levels of NO were only detected in some marine phytoplankton species, and mainly in those responsible for HABs. Considering that *S. marinoi* is a cosmopolitan bloom-forming species (Kooistra et al., 2008), a potential role for NO as an indicator of growth status and its involvement in regulating cell density during natural blooming conditions is a promising aspect worth investigating. In my experiments, NO concentrations were highest at two key steps of growth, i.e. in the early stationary and early declining phases of growth, but at very similar values ( $10.84 \pm 0.18$  r.u., and  $11.50 \pm 0.64$  r.u., respectively, Fig. II-9), while NO concentrations were lower ( $6.59 \pm 1.02$  r.u., on average) during the exponential phase of growth. When adding NO externally to *S. costatum* cultures, low concentrations are able to promote algal growth, while higher concentrations cause inhibition (Zhang et al., 2005; Zhang et al., 2003). The same was observed for the HAB species *Chaetoceros curvisetum* (Zhang et al., 2006a).

Two main enzymatic pathways are thought to be responsible for NO biosynthesis in phytoplankton, a NOS-like pathway (Kim et al., 2006) and a NR pathway (Sakihama et al., 2002). In photosynthetic eukaryotes NR plays a key role in the assimilation of nitrate ( $\text{NO}_3^-$ ) and its conversion to nitrite ( $\text{NO}_2^-$ ) before it is incorporated into biomass (Allen et al., 2005). The activity and the concentration of NR inside the cell are directly related to

the growth of marine diatoms and  $\text{NO}_3^-$  uptake (Berges and Harrison, 1995). In *S. costatum* it has been shown that depending on the nitrogen source and the supply rate, a differential activity of NR is present (Jochem et al., 2000). An increase in NR abundance and activity was detected during an upwelling simulation experiment in which *S. costatum* was exposed to a shift from  $\text{NH}_4$  to  $\text{NO}_3$  as the main nitrogen source (Jochem et al., 2000). This is thought to be ecologically relevant in terms of nitrate assimilation capacity, being *S. costatum* an important bloom-forming species in diverse marine ecosystems upon nitrate entrainment into the upper water column by upwelling, wind mixing or land runoff (Guillard and Kilham, 1977).

Besides being involved in nitrogen metabolism, NR can also mediate NO production from nitrite, even though the reduction efficiency has been estimated to be relatively low (Rockel et al., 2002). Generally, upon production, nitrite is immediately reduced to ammonium ( $\text{NH}_4^+$ ) by the enzyme nitrite reductase (NiR), which is present inside the chloroplasts of microalgae. However, if nitrite can accumulate inside the cytosol at significant concentrations, NO can also be produced by NR as a byproduct of nitrogen metabolism. This has been shown to occur under stress conditions in association to impairment of photosynthetic electron transport (Shingles et al., 1996). It is however possible to speculate that this could also happen during a condition of active growth, for instance at the beginning of the log phase of algal growth, with a higher NR activity and consequently a higher nitrite accumulation with concomitant higher NR efficiency in producing NO. This would be consistent with my results, i.e. NR dependent NO production in *S. marinoi* and higher endogenous NO levels due to active growth through NR-dependent nitrate assimilation.

Apparently, NOS is not involved in NO generation in *S. marinoi*. In particular, results obtained with the NR inhibitor ST were comparable to those obtained with the NO

scavenger cPTIO (Fig. II-10), therefore supporting the hypothesis of NR-dependent NO production, instead. On the contrary, the NOS inhibitor LNAME did not have any effect (Fig. II-10). This was probably due to a lower degree of NO production related to different cell physiological status (see Fig. II-5b,c). The fact that NOS-like activity was not evident in *S. marinoi* cells is consistent with what has been previously reported by Chung et al. (2008), that the source of intracellular NO in *S. costatum* was not found to be dependent on a NOS-like pathway. Previously, a NOS-independent NO production has also been reported in the green alga *Scenedesmus obliquus* (Mallick et al., 2000b) and in the green alga *Chlamydomonas reinhardtii* (Sakihama et al., 2002). It should be noted, however, that NOS-like activity has been found in the raphidophycean flagellate *Chattonella marina* (Kim et al., 2006) and in the green freshwater microalga *Chlorella* (Estevez and Puntarulo, 2005). This is in accordance with the recent finding that the sequences of two L-arginine-dependent NOS enzymes were characterized from two green algal species of the genus *Ostreococcus* (Foresi et al., 2010), which might suggest for the existence of a NOS enzyme in plants as well (Eckardt, 2010).

It is interesting to note that in the antarctic *Chlorella* sp. NO production is dependent upon both NR and NOS-like activity, depending on the specific stage of the exponential growth phase, with NR active at the beginning of the log phase and during the entire exponential phase and NOS later during the exponential phase of growth (Estevez and Puntarulo, 2005). This suggests that the specific growth phase as well as the intracellular availability of substrates and cofactors have a key role in the relative physiological contribution of these two enzymes to the total cellular NO. Since my experiments were all performed at the beginning of the log phase, I cannot rule out the possibility of an involvement of NOS in NO production in other growth phases.



## **CHAPTER 3**

### **Nitric Oxide (NO) and Reactive Oxygen Species (ROS)**

### **Production in Response to Polyunsaturated Aldehydes (PUAs)**

#### **3.1 NO Production in Response to PUAs**

##### **3.1.1 Introduction**

Marine diatoms are one of the most successful groups of eukaryotic photosynthetic organisms on Earth and are major players in global marine primary production by generating most of the organic carbon that is at the base of the marine food web (Armbrust, 2009). A number of different marine diatoms are known to produce polyunsaturated aldehydes (PUA) as secondary metabolites derived from fatty acid metabolism (Wichard et al., 2005b). PUAs, together with a plethora of other different metabolites derived from the same biosynthetic pathway, all belonging to oxylipins, are thought to play key roles in chemically-mediated plankton interactions (for a review see Ianora and Miralto, 2010). In a study investigating 51 cultured diatoms strains, 30% were found to be PUA producers (Wichard et al., 2005b), suggesting that they play a significant role in diatom biology and possibly in regulating biological interactions in the natural environment. Since the first identification of PUAs in marine diatoms (Miralto et al., 1999), these compounds have been demonstrated to have negative effects on copepod development and reproduction, as well as deleterious effects on different marine invertebrates (Buttino et al., 2004; Caldwell et al., 2003; Ianora et al., 2004; Romano et

al., 2003). Recently, in the sea urchin *Paracentrotus lividus*, high concentrations of the PUA decadienal have been found to induce NO production leading to apoptotic events, while at lower PUA concentrations increased NO was found to have a protective role through the activation of *hsp70* gene expression (Romano et al., 2011). PUAs also have a role in shaping marine bacterial community composition by conferring a competitive advantage to PUA-resistant bacterial groups (Balestra et al., 2011; Ribalet et al., 2008). In a benthic diatom, PUAs have been recently shown to impair adhesion to substrates and negatively affect photosynthetic efficiency (Leflaive and Ten-Hage, 2011b). Additionally, PUAs have been reported to act as allelochemicals by inhibiting the growth of different phytoplankton species, thereby possibly conveying an ecological advantage to PUA-producing phytoplankton species compared to non-producing ones (Casotti et al., 2005; Ribalet et al., 2007a). PUAs may function also as signal molecules to determine cell fate and death of diatom populations. It was speculated that a sophisticated stress surveillance system, involving nitric oxide (NO) and calcium ( $\text{Ca}^{2+}$ ), might exist in diatoms in response to PUAs. This could be a determinant of bloom termination and cell death above threshold PUA concentrations (Vardi et al., 2006).

NO is known to be a very versatile molecule involved in many different functions in animals, such as neurotransmission, vasodilation, and defence against pathogens (e.g. Moroz and Kohn, 2011; Wink and Mitchell, 1998). It is now well established that NO is also a key molecule in many physiological and defence processes in plants (Lamattina et al., 2003), fungi (Carmona et al., 2012) and bacteria (Plate and Marletta, 2012). In plants NO regulates a number of different genes, involved for instance in photosynthesis, cell death and basic cellular metabolism (for a review see Wendehenne et al., 2004). Compared to animals and higher plants, studies of NO production in marine phytoplankton are limited. NO appears to regulate physiological processes and stress

responses in the chlorophycean *Scenedesmus obliquus* (Mallick et al., 2000a; Mallick et al., 1999) and ichthyotoxicity in raphidophycean flagellates (Kim et al., 2008). In addition, NO production in the dinoflagellate *Symbiodinium* spp. induces coral bleaching (Bouchard and Yamasaki, 2009; Perez and Weis, 2006). NO has also been reported to be produced during normal growth in different phytoplankton species (e.g. Estevez and Puntarulo, 2005; Zhang et al., 2006b), suggesting a role as a growth regulation factor. In different diatom species NO has been found to play a potential role as a signalling molecule in sensing PUA-derived stress (Vardi et al., 2006), in regulating gene expression and subsequent cell death under light stress (Chung et al., 2008), in controlling adhesion of benthic species and *Ulva* zoospores to different substrates (Thompson et al., 2010; Thompson et al., 2008), and in the inhibition of biofilm formation in response to chemical stress (Leflaive and Ten-Hage, 2011a).

Vardi and co-workers (2006) exposed two diatom species to the diatom-derived aldehyde (2*E*,4*E*/*Z*)-decadienal (DECA). The authors found that the response was concentration dependent. At high concentrations DECA induced apoptotic-like cell death, whereas at low concentrations it triggered resistance to the toxic effect of this aldehyde. In both types of responses NO was found to act as a signalling molecule in driving the observed effects. The authors also investigated the role of calcium ( $\text{Ca}^{2+}$ ) in inducing NO generation. In particular, using transformed *P. tricornutum* cells expressing a calcium-sensitive photoprotein (aequorin), they were able to monitor changes in intracellular calcium in response to DECA exposure. Interestingly, they found a calcium increase following DECA treatment, suggesting that NO played a role downstream of calcium. They also reported a  $\text{Ca}^{2+}$ -dependent increase in NOS activity after exposure to the aldehyde. The significance of this paper resides in the finding that upon exposure to PUAs different types of responses might be elicited, and that NO and calcium are

involved in the resulting pathways. Later on, the same authors identified in *P. tricornutum* a gene associated to NO production, which they named *NOA1* (*Nitric Oxide-Associated 1*) (Vardi et al., 2008). This is a GTP-binding protein which belongs to the highly conserved group of YqeH proteins, and it is believed to have an important role in NO synthesis (Guo et al., 2003) and ribosome assembly (Moreau et al., 2008). In addition to a higher NO production, other effects induced by the over expression of this gene in *P. tricornutum* included reduced growth and impaired photosynthesis efficiency compared to wild-type organisms (Vardi et al., 2008). The authors found that the protein of interest was localized within the plastid, differently from the plant ortholog which is localized within the mitochondria. Exposure to DECA in PtNOA transformants resulted in an altered expression of superoxide dismutase and increased metacaspase activity, which are known to be major players in stress responses and programmed cell death pathways (Bidle and Falkowski, 2004; Wolfe-Simon et al., 2006).

The question arises as to whether PUA-producing species show different adaptive or responsive mechanisms in response to PUAs. In *S. costatum* (now *S. tropicum*) NO was found to be a key secondary messenger for the expression of a recently discovered gene encoding for a death specific protein, named *ScDSP-1* (Chung et al., 2005; Chung et al., 2008). The level of expression of *ScDSP-1* was found to increase in cultures during the late stationary phase of growth (Chung et al., 2005), as well as in cultures subject to light manipulation and cultures treated with different herbicides (Chung et al., 2008), which act as photoinhibitors by interrupting photosynthetic electron flow (Ashton and Crafts, 1981). The specific site of action of these compounds can be either at the level of photosystem I (PSI) or photosystem II (PSII). The herbicide paraquat (methyl viologen) acts by intercepting electrons at the reducing site of photosystem I, leading to the production of the free radical superoxide anion ( $O_2^-$ ) which can cause rapid loss of

chloroplastic activity (Taiz and Zeiger, 2010). On the contrary, in the case of DCMU [Diuron 3-(3,4-Dichlorophenyl)-1,1-dimethylurea], the site of action is the quinone acceptor complex in the electron transport chain between PSII and PSI (Taiz and Zeiger, 2010).

In *S. costatum* cultures transferred from high light to low light there was a net increase in *ScDSP-1* mRNA, suggesting that reduction in the electron flow might be responsible for the NO-mediated expression of this gene. Moreover, when treating cells with two different photoinhibitors, DCMU and DBMIB (2,5-dibromo-3-methyl-6-isopropylbenzoquinone), both acting at the level of PSII, the expression of the gene dramatically increased. Taken together, the results of Chung and co-workers (2008) suggest that the blockage of the electron flow between PSII and the cytochrome *b<sub>6</sub>f* was the main factor inducing an increase in NO production which then resulted in an increase in *ScDSP-1* expression. Another finding was that under high light, *ScDSP-1* expression rapidly increased with the age of the culture. However, in this case the expression of the *ScDSP-1* might have been regulated by a combination of different 'stress factors'. Nevertheless, the evident correlation between NO production and *ScDSP-1* expression reported by the authors strongly suggests that age dependent expression of *ScDSP-1* in *S. costatum* is indeed mediated by NO. These authors found that NO generation was not dependent on NOS-like activity. Given the fact that DSP is hypothesized to be a calcium regulated protein (Chung et al., 2005), it is possible that  $\text{Ca}^{2+}$  acts downstream of NO, consistent with the role of NO as a calcium mobilizing agent (Courtois et al., 2008), and in contrast to what had been suggested by Vardi et al. (2006).

NO was also recently reported to function as a signalling molecule in the benthic diatom *Seminais robusta*, as related to its ability to adhere to different substrates (Thompson et al., 2008). The authors found that NO production was negatively correlated

to adhesion to preferred hydrophobic vs. hydrophilic substrates. The authors speculated that NO controls diatom motility by signalling unsuitable surfaces thereby inducing diatoms to detach and move to a more suitable microenvironment. This was also found to hold true for the attachment of *Ulva* sp. zoospores (Thompson et al., 2010). These results suggest that NO can function as a universal signal for detecting the properties of surfaces in differential algal species. In line with these findings, recently, Leflaive and Ten-Hage (2011a) reported that the diatom-derived polyunsaturated aldehyde decadienal induces an increase in NO production in the benthic diatom *Fistulifera saprophila*, which translates into the inhibition of biofilm formation and cell motility, and also induces an increased aggregation of cells.

The main aim of the work reported in this chapter was to investigate whether PUA-producing diatoms might have evolved different adaptive mechanisms to PUA exposure with respect to non-PUA-producing ones. Previously tested diatoms were *Thalassiosira weissflogii* and *Phaeodactylum tricornutum* (Vardi et al., 2006), which do not produce PUAs, and which have never been reported in the natural environment at high concentrations, therefore limiting the ecological significance to be extrapolated from these results. Instead, the diatom model species chosen for this study, *Skeletonema marinoi* is a bloom-forming marine diatom spread worldwide (Kooistra et al., 2008), which produces high amounts of three main PUAs: 2,4-heptadienal, 2,4-octadienal, and 2,4,7 octatrienal (Ribalet et al., 2007b). The work presented here focuses on NO dynamics in response to different stress factors, in particular to PUAs exposure and to light stress simulated by the use of two photoinhibitors: DCMU, which has been previously related to NO production in *S. costatum* (Chung et al., 2008), and paraquat. . Additionally, the comparative analysis of NO-production pathways between a PUA-producing (*S. marinoi*) and a non-PUA-producing (*P. tricornutum*) diatom is presented.

### 3.1.2 Materials and Methods

#### 3.1.2.1 Cultures

For *S. marinoi* see Materials and Methods 2.1.2.1, Chapter 2. *Phaeodactylum tricornutum*, strain RCC69, was obtained from the Roscoff Culture Collection (Roscoff, France). Natural filtered seawater (FSW) from the Bay of Naples, amended with f/2 nutrients, was used as growth medium. *S. marinoi* was grown in 2 L polycarbonate bottles (Nalgene, Termo Fisher Scientific, USA) with air bubbling, except for cultures for the PUAs long term exposure experiment where *S. marinoi* was grown in polystyrene flasks (Corning Inc., NY, USA). *P. tricornutum* was cultured in polystyrene flasks (Corning Inc., NY, USA). For culturing conditions see Materials and Methods 2.1.2.1, Chapter 2. Cultures were kept in the exponential phase of growth for at least 6 generations before performing the experiments.

#### 3.1.2.2 Preparation of Chemicals

For *in vivo* NO detection the fluorescent NO-sensitive dyes DAF-FM DA and the nitric oxide sensor (intracellular) kit “NO-ON” (CuFL) were used (see Materials and Methods 2.1.2.2, Chapter 2). The PUAs used were: 2E,4E-heptadienal (HEPTA), 2E,4E-octadienal (OCTA), and 2E,4E-decadienal (DECA) and a combination of OCTA and HEPTA (MIX) (all from Sigma Aldrich Inc., Milan, Italy). The MIX was composed by the two PUAs in the same proportion as produced naturally by *S. marinoi* in culture (Ribalet et al., 2007b). Working solutions of PUAs were prepared by diluting the stock in absolute methanol (MeOH) (JT Baker, Phillipsburg, NJ, USA) at room temperature. The effective PUA concentration of the working solution was assessed spectrophotometrically before inoculation at a wavelength of 274 nm using a specific molar absorption

coefficient of 31000 (Pippen and Nonaka, 1958). The concentrations used were chosen accounting for multiples of the EC50 concentration for growth at 24 h, as reported in Ribalet et al. (2007a) for *S. marinoi*, and Vardi et al. (2006) for *P. tricornutum*.

The NO scavenger carboxy-PTIO (cPTIO) (Enzo Life Sciences, Vinci, Italy) was used at a final working concentration of 100  $\mu$ M, dissolved in filtered sea water (FSW), and incubated for 20 min. 100 mM stock solution of the NO donor DEA NONOate (DEANO) (Vinci Biochem, Vinci, Italy) was prepared in 0.01 M NaOH. Final optimal working concentration was set at 0.1 mM, and samples were incubated for 20 min. Tests were performed in order to verify that the pH in the growth medium was not being altered by adding NaOH (data not shown).

The photoinhibitors used were Diuron 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) and methyl viologen dichloride hydrate (Paraquat) (both from Sigma-Aldrich, Milan, Italy). A stock solution of 10 mM in 100% Ethanol (EtOH) (Sigma-Aldrich, Milan, Italy) was freshly prepared just before the experiments. Final working concentrations were 50  $\mu$ M for DCMU and 100  $\mu$ M for Paraquat. Samples were incubated with photoinhibitors for 4 hours before the measurements.

#### 3.1.2.3 Flow Cytometry

See Material and Methods 2.1.2.3, Chapter 2.

#### 3.1.2.4 Statistical Analysis

Statistical significance with respect to control values was evaluated with the Student's *t*-test using the Excel spreadsheet (Microsoft Office).



### 3.1.3 Results

#### 3.1.3.1 NO dynamics in response to PUAs in *S. marinoi*

When exposed to DECA, no increase in green fluorescence from DAF-FM DA was observed, indicating absence of NO production. Instead, a clear decrease in the production of NO relative to the control was evident, which was DECA concentration dependent (Fig. III-1). This was probably due to a consumption of basal levels of NO and a parallel inhibition of new production, as also confirmed by the lack of effect of the scavenger cPTIO (Fig. III-1 a). The average decrease in NO production after 180 min exposure to DECA (averaged among all the different DECA concentrations tested) was 52% (ranging from 20% to 85%), the highest observed of all the PUAs tested. Reduced NO production was significant for all the DECA concentrations tested (0.05  $\mu$ M:  $p < 0.05$ ; 0.1  $\mu$ M :  $p < 0.05$ ; 1  $\mu$ M :  $p < 0.01$ ; 5  $\mu$ M :  $p < 0.001$ ; 33  $\mu$ M  $p < 0.001$ ; 33  $\mu$ M :  $p < 0.001$ . For all concentrations,  $n = 3$ ). A similar trend of NO decrease was observed after inoculation with OCTA and HEPTA (Fig. III-1 b and III-1 c, respectively), but the decreasing trend was less evident. However, the average decrease for all tested concentrations was slightly stronger with HEPTA than with OCTA (40% average decrease at 180 min, ranging from 10% to 68%, vs. 31%, ranging from 0% to 47%, respectively). At 180 min a statistically significant reduction in NO production was evident starting from OCTA 1  $\mu$ M (1  $\mu$ M, 10  $\mu$ M and 20  $\mu$ M :  $p < 0.05$ ; 10  $\mu$ M :  $p < 0.01$ . For all concentrations,  $n = 3$ ). For HEPTA, NO reduction at 180 min was less significant for 1  $\mu$ M and 5  $\mu$ M ( $p < 0.05$ ,  $n = 3$ ) compared to 10  $\mu$ M, 20  $\mu$ M and 40  $\mu$ M ( $p < 0.01$ ,  $n = 3$ ). With the MIX, values were similar to the ones elicited by OCTA (30% average decrease at 180 min, ranging from 13% to 49%) (Fig.III-1 d), and a statistically significant reduction in NO was evident only at 10  $\mu$ M OCTA plus 14  $\mu$ M HEPTA and

20  $\mu$ M OCTA plus 28  $\mu$ M HEPTA ( $p < 0.01$ ,  $n = 3$ ). A clearer trend in NO decrease between the different total concentrations was observed at 180 min with the MIX treatment when compared to the single PUAs. No synergistic effect of the two PUAs inoculated together was evident in our experiments, since the effect was never higher than with OCTA or HEPTA alone.

NO production probed with CuFL confirmed the lack of NO production in response to PUAs in *S. marinoi*. Cultures were exposed to different concentrations of OCTA, and although no enhancement in NO production was evident, with CuFL the trend in the decrease of endogenous NO levels was less clear compared to DAF. In particular, with OCTA, the average decrease at 120 min was 24% (ranging from 17% to 34 %), but without a clear trend (Fig. III-2). The highest decrease was observed at 120 min with 0.05  $\mu$ M OCTA ( $34\% \pm 5.2\%$ ;  $p < 0.05$ ,  $n = 3$ ).

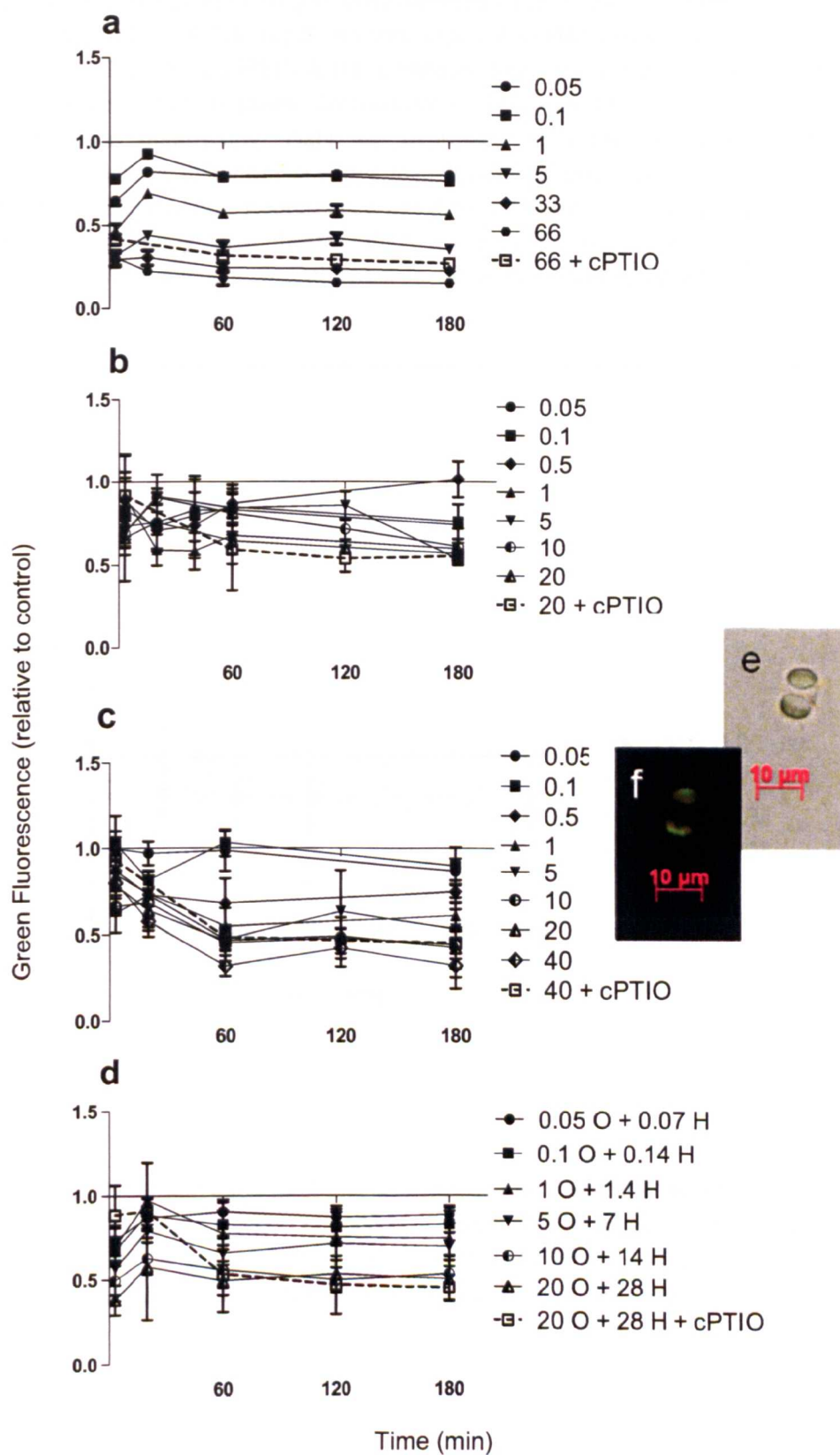


Figure III- 1: Caption on opposite page.

Figure III- 1 NO production in *S. marinoi* exposed to different concentrations of different PUAs. Values in the legend refer to  $\mu\text{M}$  concentrations. (a) *S. marinoi* exposed to DECA; (b) *S. marinoi* exposed to OCTA; (c) *S. marinoi* exposed to HEPTA; (d) *S. marinoi* exposed to the MIX [i.e. OCTA (O) and HEPTA (H) combined together]; (e) *S. marinoi* cells observed with light microscopy and (f) green fluorescence of DAF-loaded *S. marinoi* cells observed with epifluorescence microscopy. Data are expressed in terms of NO-DAF derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates. Each experiment was performed at least twice. cPTIO represents a NO scavenger, inoculated with the highest concentration of PUA tested in each experiment.

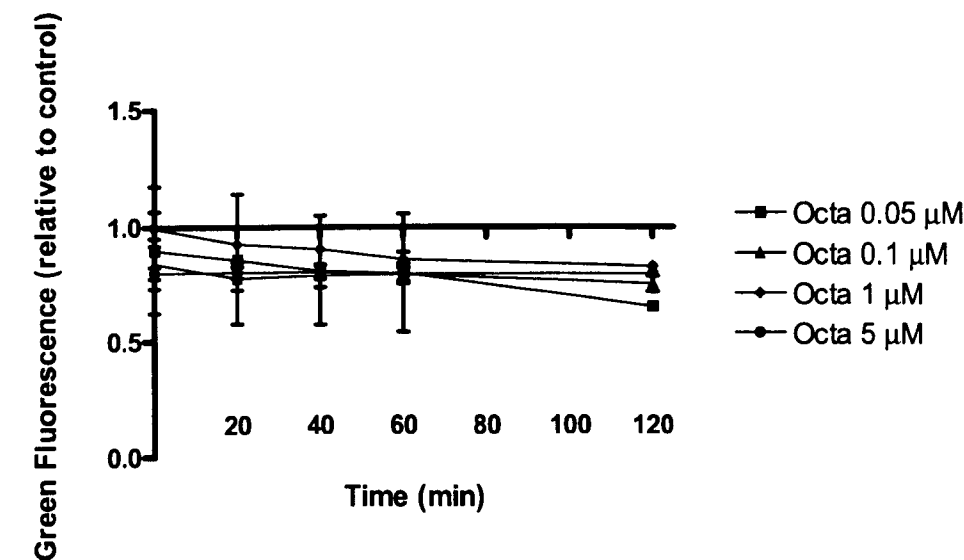


Figure III- 2: NO production in *S. marinoi* exposed to different concentrations of OCTA. Data are expressed in terms of NO-CuFL derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates.

### 3.1.3.2 NO dynamics in response to PUAs in *S. marinoi* over long term exposure

In order to verify if in *S. marinoi* PUAs might trigger NO production over the long term, cultures were exposed to different OCTA concentrations up to 24 h. Results showed that NO levels always remained lower compared to the control. In particular, a decreasing trend was evident at 12 h, when the highest decrease in NO levels was found with OCTA 1  $\mu$ M ( $21\% \pm 3\%$  decrease;  $p < 0.001$ ,  $n = 3$ ) (Fig. III-3). This confirms that PUAs are not able to elicit an increase in NO production in *S. marinoi*. Results with the NO scavenger cPTIO confirmed DAF specificity for NO.

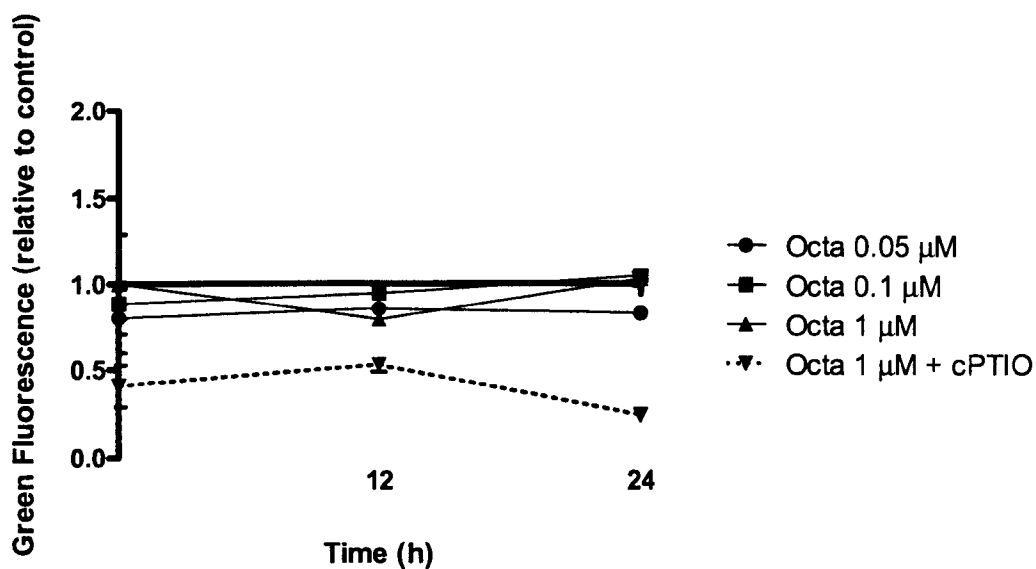


Figure III- 3: NO production in *S. marinoi* exposed to different concentrations of OCTA. Data are expressed in terms of NO-DAF derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates. cPTIO represents a NO scavenger, inoculated with the highest concentration of PUA tested.

### *3.1.3.3 NO production in response to light stress*

DCMU and paraquat represent two types of herbicides that act by interrupting photosynthetic electron flow, thereby simulating photoinhibition (Ashton and Crafts, 1981). Paraquat acts by accepting electrons from the early acceptors of photosystem I (PSI), while DCMU acts by blocking the electron flow at the quinone acceptors of photosystem II (PSII) (Taiz and Zeiger, 2010). Paraquat did not have any significant effect in eliciting NO production (1.2 times the control), while DCMU had a stronger effect inducing NO production ( $3.5 \pm 0.7$  times the control;  $p < 0.01$ ,  $n = 3$ ) (Fig. III-4). The lower increase in NO production detected with the NO scavenger cPTIO, coupled with DCMU, confirmed DAF reliability for NO detection ( $2.4 \% \pm 0.5 \%$  times the control;  $p < 0.05$ ,  $n = 3$ ) (Fig. III-4). Results with EtOH confirmed that the solvent did not have any effect on the NO-DAF-derived green fluorescence (Fig. III-4).

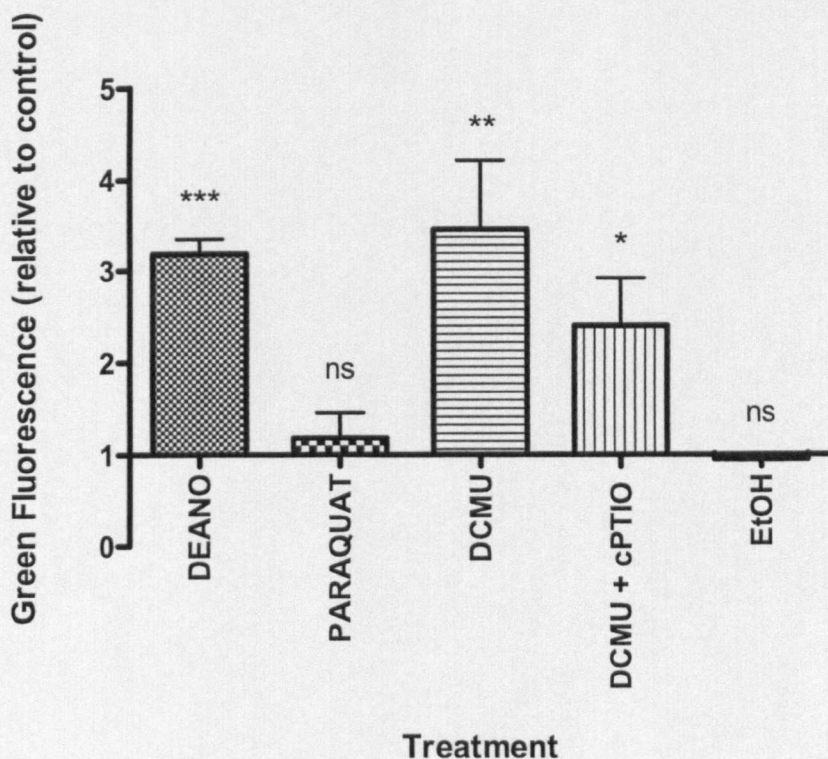


Figure III- 4: NO production in *S. marinoi* cultures treated with the two different photoinhibitors DCMU and Paraquat; treatments with DEANO (NO donor) and cPTIO (NO scavenger) represent the positive and negative control, respectively. EtOH was tested to exclude any interference of the solvent with NO-DAF-derived green fluorescence. Data are expressed in terms of NO-DAF derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates. Each experiment was performed at least twice. Statistical significance was evaluated with the Student's *t*-test with respect to control values. \*\*\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*  $p < 0.001$ .  $n = 3$ . ns = not significant.



#### 3.1.3.4 Nitric Oxide (NO) in *Phaeodactylum tricornutum*

In order to exclude artefacts in the experimental setup, and to convalidate the results pointing to a lack of NO increase upon PUA exposure in *S. marinoi*, I replicated the experiments with *P. tricornutum* as reported in Vardi et al. (2006), but also added OCTA to the treatments, in order to expand the comparison between the two species exposed to this PUA, so to compare the observed responses of *S. marinoi*. In *P. tricornutum* DECA induced an increasing trend in NO production (Fig. III-5 a), as reported by Vardi et al. (2006). In addition, when *P. tricornutum* was exposed to OCTA, instead, no NO production was elicited and a decreasing trend of DAF-FM-NO fluorescence was observed (Fig. III-5 b), similarly to *S. marinoi*, suggesting a differential response of *P. tricornutum* to different PUAs.

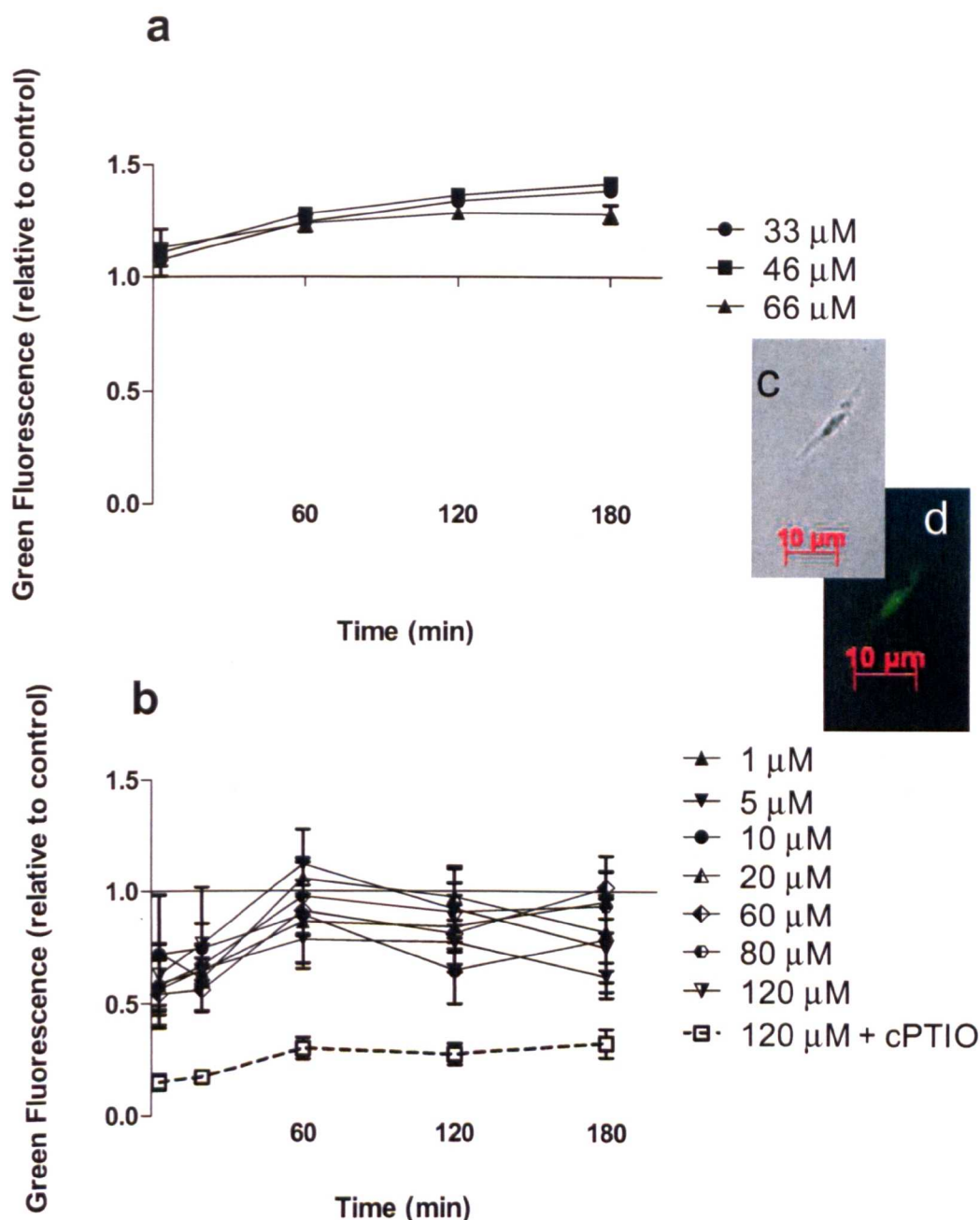


Figure III- 5: NO production in *P. tricornutum* exposed to DECA (a) and OCTA (b). (c) *P. tricornutum* cells observed with light microscopy and (d) green fluorescence of DAF-loaded *P. tricornutum* cells observed with epifluorescence microscopy. Data are expressed in terms of NO-DAF derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates. Each experiment was performed at least twice. cPTIO represents a NO scavenger, inoculated with the highest concentration of PUA tested.

### 3.1.4 Discussion

The fact that it was not possible to detect an increase in NO production in *S. marinoi* exposed to PUAs is surprising as NO appears to be involved in different stress responses in marine macro-and microalgae (Zhang et al., 2006b; Bouchard and Yamasaki, 2009; Ross et al., 2006), including diatoms (Vardi et al., 2006). The other diatom *P. tricornutum*, instead, responds to DECA with an increase in NO (Vardi et al., 2006), suggesting that different stress-signalling pathways are elicited by this PUA in the two species, pointing to a species-specific type of response. Indeed, in *P. tricornutum* a gene associated to NO production, designated PtNOA, has been identified (Vardi et al., 2008), while no such type is available yet for *S. marinoi*. Exposure to DECA in PtNOA transformants resulted in an altered expression of superoxide dismutase and increased metacaspase activity (Vardi et al., 2008), which are known to be major players in stress responses and programmed cell death pathways (Bidle and Falkowski, 2004).

Another interesting aspect is that while *S. marinoi* does not produce NO in response to any PUAs, *P. tricornutum* does so only for DECA and not for the other PUAs tested, likely to indicate the existence of a PUA-specific type of response in the latter. Despite being the most used PUA in toxicological experiments, DECA is not the most common PUA present in marine phytoplankton. In a survey of 51 species of marine diatoms, DECA was the least detected PUA (Wichard et al., 2005a) and this also appears to be the case in natural seawater (Vidoudez et al., 2001a). Indeed, *P. tricornutum* is reported not to produce any PUAs (Wichard et al., 2005a). In nature, DECA is known to be produced by the bloom-forming phytoplankter *Phaeocystis pouchetii* (Hansen et al., 2004, Hansen and Eilertsen, 2007). It is possible that the response to DECA in *P. tricornutum* is elicited by DECA's stronger activity and toxicity, while the response to OCTA might be more related to its role as a signal in nature and to its natural occurrence. This would support

the hypothesis that PUAs with a less strong reactivity and commonly present in nature, such as OCTA and HEPTA, can act as infochemicals for diatoms. The strongest effect elicited by DECA with respect to the other PUAs might be due to its longer carbon chain (Adolph et al., 2003), as suggested also by other authors (e.g. Casotti et al., 2005; Ianora and Miralto, 2010).

It is important to point out that I not only did not observe any increase in NO in response to PUA exposure in *S. marinoi*, but indeed found a decrease in NO-related fluorescence. This was a real decrease, as confirmed by the weak effect of the NO scavenger cPTIO. This also suggests an impairment of the physiological NO production, probably related to growth inhibition. In the antarctic *Chlorella* sp. an increase in NO production triggered the passage of the culture into the exponential phase of growth, suggesting that NO could be a key growth regulatory molecule (Estevez and Puntarulo, 2005). Additionally, a correlation was found between NO production and growth in different marine phytoplankton species, including *Skeletonema costatum*, with a peak in NO occurring right before highest cell densities (Zhang et al., 2006b). In line with these findings, I also observed increasing NO values in *S. marinoi* during the log phase of growth (See Chapter 2.2). Therefore, the observed decrease in NO in *S. marinoi* upon exposure to PUAs may be also due to the growth-inhibiting effect of PUAs reported by other authors (Casotti et al., 2005; Ribalet et al., 2007a). Many studies testing PUAs toxicity on different organisms have used singles PUAs rather than a combination of them (e.g. Romano et al., 2011; Leflaive and Ten-Hage, 2011a; Bisignano et al., 2001). It is worth noting, however, that when used together, PUAs showed both a synergistic effect in natural bacterioplankton assemblages (Balestra et al., 2011), and no additive effect in a *S. marinoi* culture during different phases of growth (Vidoudez and Pohnert,

2008). In the case of my experiments I could not observe an added effect of OCTA and HEPTA when inoculated together.

When *P. tricornutum* is exposed to DECA, NO levels increase and appear to be involved in a signalling response (Vardi et al., 2006). Instead, when it is exposed to OCTA, a decrease in NO is observed, similar to what happens in *S. marinoi*. This might be related to *P. tricornutum* higher growth rates in culture and thus possible higher physiological NO levels when compared to *S. marinoi*, which was also confirmed by the relatively stronger scavenging effect of cPTIO (Fig. III-5). The difference in NO physiology in these two diatom species, as well as possibly the difference in NO biosynthetic pathways, may underlie their differential response to PUAs and their dramatically different growth patterns and capabilities in culture, opposite to their ecological occurrence and metabolism in the natural environment.

The experiments on light stress showed that in *S. marinoi* the PSII photoinhibitor DCMU elicited a 3.5 fold increase in NO production relative to control, while the photoinhibitor paraquat, which acts at the level of PSI, did not (Fig. III-4). Also in the congeneric *Skeletonema costatum* (now replaced as *S. tropicum*) NO production was elicited by the PSII inhibitors DCMU and DBMIB, but not by paraquat (Chung et al., 2008). DCMU and paraquat represent two types of herbicides that act by interrupting photosynthetic electron flow (Ashton and Crafts, 1981). Paraquat acts by accepting electrons from the early acceptors of photosystem I (PSI), while DCMU acts by blocking the electron flow at the quinone acceptors of photosystem II (PSII) (Taiz and Zeiger, 2010). More specifically, paraquat mode's of action is based on the interception of electrons between the bound ferredoxin acceptors and NADP, and then on the reduction of oxygen to  $O_2^-$ , leading to impaired chloroplastic activity. On the other hand, DCMU competes for the binding site of plastoquinone at the quinone acceptor complex between

PSII and PSI, blocking electron flow and inhibiting photosynthesis (Taiz and Zeiger, 2010). My results confirm previous studies conducted on different microalgae, that have also reported that blocking the PS-II electron flow by means of lack of light or DCMU exposure, induce elevated intracellular NO concentrations (Sakihama et al., 2002; Mallick et al., 1999). Recently, an increase in NO production under conditions of high light stress has been linked to the protective role of NO as a signalling molecule in activating antioxidant enzyme biosynthesis (Xu et al., 2010). In my experiments, the observed decrease in NO production indicates that in PUA-exposed *S. marinoi* and in OCTA-exposed *P. tricornutum*, NO did not have a protective role against chemical stress. It can be speculated that if NO has a protective role, then the lack of NO production might imply a down-regulation of genes involved in the stress response to PUAs. Other types of stress that have been associated to NO production, both in plant and algal systems, include UVB irradiation (Krasylenko et al., 2012; Tossi et al., 2012), viral infections (Sarkar et al., 2010; Fu et al., 2010; Danci et al., 2009), heat stress associated to coral bleaching (Bouchard and Yamasaki, 2009), injury (Ross et al., 2006) and change in different environmental factors such as temperature and salinity in microalgae (Zhang et al., 2006b).

In conclusion, considering that a different chemical stress factor, i.e. PUA exposure, was unable to induce any increase in NO production in *S. marinoi*, in this diatom NO production appears to be also stress-specific.

## 3.2 ROS Production in *S. marinoi* in Response to PUAs

### 3.2.1 Introduction

Reactive Oxygen Species (ROS), such as superoxide radical, hydrogen peroxide and hydroxyl radical, represent natural byproducts of cell metabolism. Oxidative stress occurs when the metabolic level of ROS exceeds the cell elimination capacity through a series of both enzymatic and non-enzymatic defence pathways. The generation of oxygen radicals is an important element of the general stress response in marine organisms that constantly need to cope with a wide variety of stress factors, including heat, UV radiation, different chemicals and, in general, any change in environmental conditions (Lesser, 2006). Early studies conducted on the dinoflagellate *Peridinium gatunense* revealed that CO<sub>2</sub> limitation could enhance ROS formation, inducing cell death through programmed cell death (PCD) (Vardi et al., 1999). Generation of oxygen radicals has also been reported to control apoptotic cell death in yeast (Madeo et al., 1999). Other stress factors that have been reported to lead to an increase in oxidative stress in phytoplankton include viral infection (Evans et al., 2006) elevated levels of pH and iron limitation (Liu et al., 2007), prolonged darkness (Bouchard and Purdie, 2011), as well as cadmium (Jamers et al., 2009) and paraquat exposure (Rioboo et al., 2011). Additionally, ultraviolet-B (UV-B) radiation has been often associated to an increase in ROS production. This has been shown for cyanobacteria (He and Hader, 2002), the microalga *Chlorella vulgaris* (Malanga and Puntarulo, 1995), the embryonic stages of the brown seaweed *Fucus spiralis* (Rijstenbil et al., 2000), and the marine diatom *Thalassiosira pseudonana* (Rijstenbil, 2002), where hydroxyl radicals were found to trigger lipid peroxidation. Moreover, in the symbiotic dinoflagellate *Symbiodinium bermudense*, both UV and high temperatures were found to induce oxidative stress as well as inhibition of photosynthesis (Lesser, 1996). Exposure to heavy metals has also been shown to induce ROS production

in different algal species (Pinto et al., 2003; Knauert and Knauer, 2008). It is also interesting to note that, as opposed to NO, the reactive oxygen species H<sub>2</sub>O<sub>2</sub> has not been found to be involved in the toxicity derived from an harmful algal bloom (HAB)-forming raphidophyte (Twiner et al., 2001). An increase in ROS cellular levels has also been associated to the exposure of macro and microalgae to alleochemical compounds and toxins, such as a thiol protease excreted by ageing cells (Vardi et al., 2007), the cyanobacterial toxin nodularin (Pflugmacher et al., 2010), and macrophytes-derived allelochemicals (Wang J. et al., 2011). In the green macroalga *Dasycladus vermicularis* NO was found to be involved in the wound response concomitantly with hydrogen peroxide (Ross et al., 2006). Moreover, the respective activation was found to be partly co-regulated, supporting the conclusion that a signalling relationship might exist between ROS and reactive nitrogen species (RNS), as previously suggested in algae and plants (Barros et al., 2005; Neill et al., 2002b).

In the red macroalga *Furcellaria lumbricalis* exposed to the cyanobacterial toxin nodularin, a significant increase of the two antioxidative enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) has been reported (Pflugmacher et al., 2010), suggesting that an antioxidant type of response was being activated. In terms of cellular protection to PUA-derived stress, it has been previously reported that when the marine diatom *Thalassiosira weissflogii* was exposed to PUA, an increase in the photoprotective xanthophyll pigments was observed, suggesting that they play a role as antioxidants independently from light (Casotti et al., 2005). Additionally, in the freshwater microalga *Haematococcus pluvialis* an accumulation of the carotenoid astaxanthin during the encystment process triggered by paraquat exposure has been observed (Rioboo et al., 2011). The authors also reported that the accumulation of astaxanthin showed a negative



linear relationship with ROS levels, indicating that astaxanthin could have a role in coping with oxidative stress (Rioboo et al., 2011).

The aim of the following experiments was to investigate ROS dynamics in response to PUAs in *S. marinoi*, as well as the role of ROS in the regulation of stress and the protective responses in *S. marinoi*, which might underlie the ecological success of this species and the possible evolution of its adaptive responses to chemical cues in general. In terms of ROS production, two different fluorescent, ROS-sensitive dyes were used, DHR123, which was tested with all of the PUAs, and H<sub>2</sub>DCFDA, which was used only in combination with OCTA, since it was the PUA that gave the strongest response in ROS production assessed with DHR123. Additionally, growth rate, pigment content, photosynthetic efficiency and non-photochemical quenching (NPQ) have been measured in order to investigate potential physiological responses downstream of ROS (and RNS) production.

Table III- 1: List of literature reporting ROS production in algal species

Type of stress inducing ROS production	Organism	Reference
CO <sub>2</sub> limitation	<i>Peridinium gatunense</i>	Vardi et al., 1999
Elevated pH and iron limitation	<i>Chattonella marina</i>	Liu et al., 2007
Viral infection	<i>Hemilania huxleyi</i>	Evans et al., 2006
Prolonged darkness	<i>Microcystis aeruginosa</i>	Bouchard and Purdie, 2011
Cadmium	<i>Chlamydomonas reinhardtii</i>	Jamers et al., 2009
Paraquat	<i>Haematococcus pluvialis</i>	Rioboo et al., 2011
UV-B	Cyanobacteria <i>Chlorella vulgaris</i> <i>Fucus spiralis</i> <i>Thalassiosira pseudonana</i>	He and Hader, 2002 Malanaga and Puntarulo, 1995 Rijstenbil et al., 2000 Rijstenbil, 2002
UV and high temperature	<i>Symbiodinium bermudense</i>	Lesser, 1996
Heavy metals	Different algal species (review) <i>Pseudokirchneriella subcapitata</i> and <i>Chlorella vulgaris</i>	Pinto et al., 2003 Knauert and Knauer, 2008
Allelochemical compounds and toxins	<i>Peridinium gatunense</i> <i>Furcellaria lumbricalis</i> <i>Microcystis aeruginosa</i> and <i>Pseudokirchneriella subcapitata</i>	Vardi et al., 2007 Pflugmacher et al., 2010 Wang J. et al., 2011
Physical injury	<i>Dasycladus vermicularis</i>	Ross et al., 2006

### 3.2.2 Materials and Methods

#### 3.2.2.1 Cultures

For *S. marinoi* see Materials and Methods 3.1.2.1, Chapter 3.

#### 3.2.2.2 Preparation of Chemicals

For the detection of Reactive Oxygen Species (ROS) *in vivo* two fluorescent ROS-sensitive dyes were used: Dihydrorodamine 123 (DHR123, Molecular Probes, Leiden, NL) (5 mM stock in DMSO) and 5-6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H<sub>2</sub>DCFDA) (Molecular Probes, Leiden, NL). The final working concentrations were set experimentally (see Results 3.2.3.1) and resulted to be 10  $\mu$ M for DHR123 and 20  $\mu$ M for CM-H<sub>2</sub>DCFDA. Samples were inoculated with either DHR123 or CM-H<sub>2</sub>DCFDA together with PUAs in the dark at RT. Green fluorescence from both dyes was assessed by flow cytometry immediately after dye and PUAs inoculation as time 0. Staining was observed to be immediate (data not shown) and therefore no incubation time was applied. The following data point was taken after 20 minutes.

For preparation of PUAs and the NO scavenger cPTIO see Materials and Methods 3.1.2.2, Chapter 3. As a negative control for ROS, a 0.5 M stock solution of the ROS scavenger 4-Hydroxy-TEMPO (Tempol) in filtered seawater (FSW) was used. Final working concentration was 5 mM. Samples were incubated for 20 min with the scavenger cPTIO together with the PUA before addition of the dye and measurement by flow cytometry. As a ROS donor 30 wt. % hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Sigma-Aldrich, Milan, Italy) was used. A pre-dilution of 1/200 of H<sub>2</sub>O<sub>2</sub> was prepared and 50  $\mu$ L were added to 1

mL sample and green fluorescence was assessed by flow cytometry directly after H<sub>2</sub>O<sub>2</sub> addition.

### 3.2.2.3 Flow Cytometry

For Flow Cytometry see Materials and Methods 2.1.2.3, Chapter 2.

### 3.2.2.4 Pigments and Photosynthetic Performance

*S. marinoi* cultures were exposed to different concentrations of OCTA up to 48 h. For pigment concentration analysis, 10 mL of culture were filtered through 47-mm GF/F filters (Whatman, Maidstone, UK) and immediately frozen in the dark at -80°C. Pigments were analyzed by high performance liquid chromatography (HPLC) (Hewlett Packard, series 1100, Kennett Square, PA, USA). A 3- $\mu$ m C8 BDS column (100 x 4.6 mm) was used, and the mobile phase was composed of two solvent mixtures: A, methanol/aqueous ammonium acetate, 70:30 vol/ vol, and B, methanol. The gradient between the two solvents was programmed following Vidussi et al. (1996) as follows: 75% A (0 min), 50% A (1 min), 0% A (15 min), 0% A (18.5 min), 75% A (19 min). Pigments were detected at 440 nm using a photodiode array detector (model DAD series 1100, Hewlett Packard), which gives the 400- to 700-nm spectrum for each detected pigment. A fluorometer (series 1100, Hewlett Packard) allowed the detection of fluorescent pigments, with a 410 nm excitation wavelength and a 665 nm emission wavelength. Identification and quantification of single pigments were realized using chlorophyll (chl) and carotenoid standards obtained from the VKI (Water Quality Institute) International Agency for 14C Determination (Horsholm, Denmark). Pigment filters were extracted in 100% methanol (Sigma-Aldrich, Milan, Italy) and 500  $\mu$ L of 1 mol x L<sup>-1</sup> ammonium acetate (final concentration 0.33 mol x L<sup>-1</sup>) was added to the 1 mL pigment extract 5 min

before the analysis. HPLC analysis were performed under the supervision and training of Dr. C. Brunet (Stazione Zoologica di Napoli, IT). To assess the effects of PUAs on photosynthetic activity, experiments were conducted measuring minimum (dark-adapted) fluorescence ( $F_0$ ) and maximum fluorescence ( $F_m$ ) after a saturating light pulse using a Phyto-PAM fluorometer (Heinz Walz GmbH, Effeltrich, Germany). PSII quantum efficiency, or maximum photochemical yield values,  $F_v/F_m$ , defined as  $(F_m - F_0)/F_m$  (Schreiber et al., 1986) were determined after a dark adaptation period of 15 min. High  $F_v/F_m$  values indicated that the cells were in good condition (absence of growth limitation), whereas a decrease of  $F_v/F_m$  was considered an indicator of physiological stress (Sakshaug et al., 1997). Non-photochemical quenching (NPQ) involves protective non-photochemical mechanisms that quench singlet-excited chlorophylls and dissipate excess excitation energy as heat. Among the different sources of NPQ development, diatoxanthin synthesis (photoprotective xanthophyll pigment), is one of the most important. Phyto-PAM measurements ( $F_v/F_m$  and NPQ) were performed by Dr. C. Brunet in the framework of a scientific collaboration.

#### *3.2.2.5 Statistical Analysis*

Statistical significance with respect to control values was evaluated with the Student's *t*-test using the Excel spreadsheet (Microsoft Office).

### 3.2.3 Results

#### 3.2.3.1 Protocol setup

In order to assess the optimal loading concentration for DHR123 and CM-H<sub>2</sub>DCFDA different concentrations of both dyes were tested. DHR123 is an uncharged and nonfluorescent reactive oxygen species (ROS) dye that can passively diffuse across membranes where it is oxidized to cationic rhodamine 123 which exhibits green fluorescence (Johnson et al., 1980). CM-H<sub>2</sub>DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields a fluorescent derivative that is trapped inside the cell, thus avoiding leakage (West et al., 2011).

The optimal final concentrations were set at 10  $\mu\text{M}$  for DHR123 and 20  $\mu\text{M}$  for CM-H<sub>2</sub>DCFDA (Fig. III-6, a and b, respectively). Cell concentrations were kept constant at  $1.5 - 2 \times 10^5 \text{ cell mL}^{-1}$ . Experiments were always conducted during the exponential phase of growth.

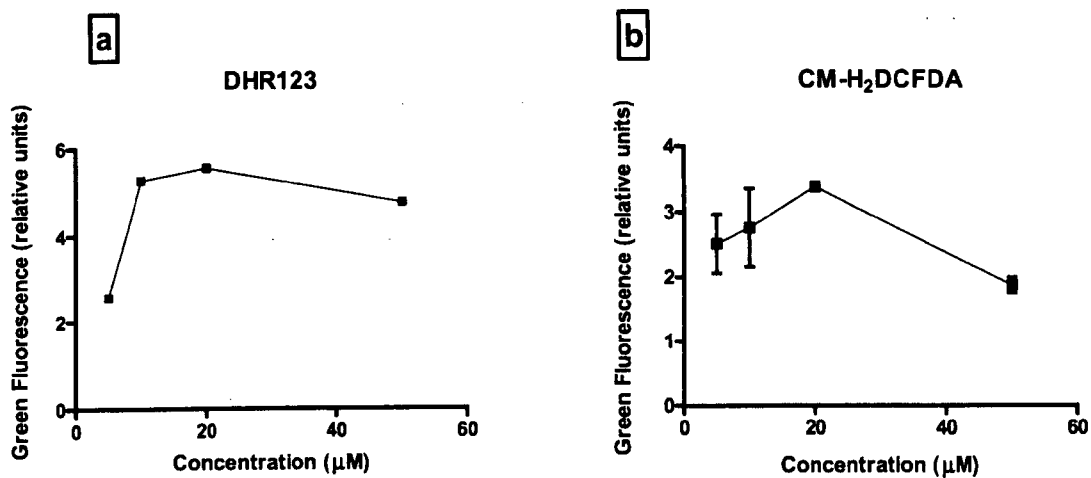


Figure III- 6: Calibration curve showing the relationship between DHR123 (a) and CM-H<sub>2</sub>DCFDA (b) concentration and ROS-derived green fluorescence. Data are means of triplicates  $\pm$  standard deviation (SD).

### 3.2.3.2 ROS production in response to PUAs in *S. marinoi* assessed with DHR123

When elicited with DECA, *S. marinoi* did not show any increase in ROS (Fig. III-7 a); the opposite was true when the other PUAs and the MIX were used (Fig. III-7 b-d), when a peak appeared within 20 min after exposure. Tests were performed to verify that no higher increase in ROS production occurred before 20 min after exposure (data not shown). The highest increase in ROS was elicited by 20  $\mu$ M OCTA (2.9 times the control,  $p < 0.001$ ,  $n = 3$ ) Fig. III-7 b), as compared to 20 and 40  $\mu$ M HEPTA (1.8 times the control,  $p < 0.01$ ,  $n = 3$ ) Fig. III-7 c). The increase started at concentrations equal or higher than 5  $\mu$ M OCTA (i.e. half the EC<sub>50</sub>, 1.6 times the control,  $p < 0.05$ ,  $n = 3$ ). When inoculated together, OCTA and HEPTA elicited an increase comparable to OCTA alone (2.9 times the control with 20  $\mu$ M OCTA plus 28  $\mu$ M HEPTA,  $p < 0.001$ ,  $n = 3$ ) (Fig. III-7 d), with no additive effect observed. In all these experiments, the scavenger Tempol induced a lower DHR-ROS green fluorescence than the treated samples, confirming that the observed increase was really due to ROS production (Fig. III-7 b-d).



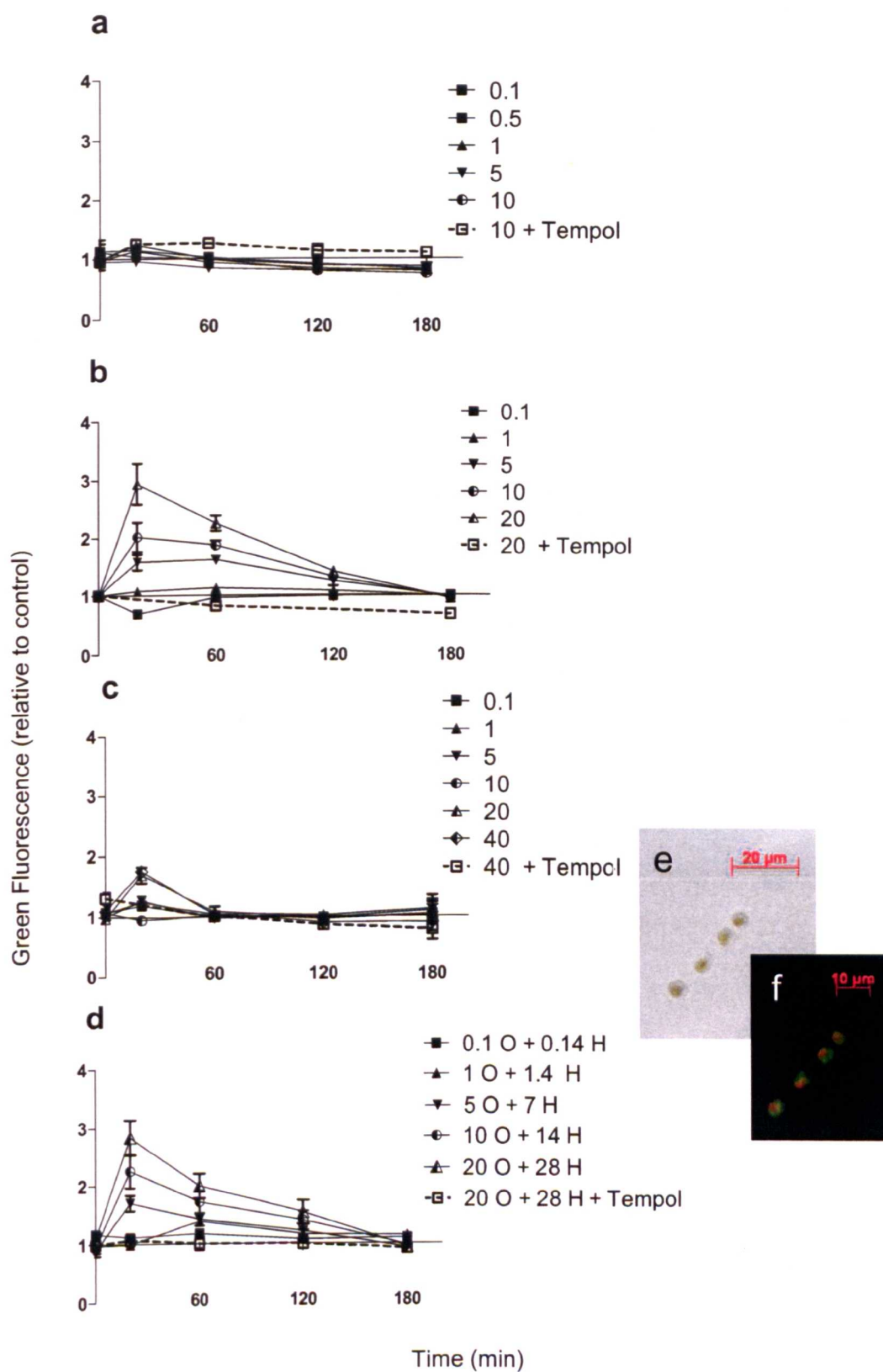


Figure III- 7: Caption on opposite page.

Figure III-7: ROS production in *S. marinoi* exposed to different concentrations of different PUAs. Values in the legend refer to  $\mu\text{M}$  concentrations. (a) DECA; (b) OCTA; (c) HEPTA; (d) MIX [i.e. OCTA (O) and HEPTA (H) combined together]. (e) *S. marinoi* cells observed with light microscopy and (f) green fluorescence of DHR123-loaded *S. marinoi* cells observed with epifluorescence microscopy. Data are expressed in terms of ROS-DHR123 derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates. Each experiment was performed at least twice. Tempol represents a ROS scavenger, inoculated with the highest concentration of PUA tested in each experiment.

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### 3.2.3.3 ROS production in response to PUAs assessed with CM-H<sub>2</sub>DCFDA

ROS production in response to OCTA, which elicited the highest ROS production measured with DHR123, was assessed also with the fluorescent dye CM-H<sub>2</sub>DCFDA. Results confirmed an increase in ROS production in OCTA exposed *S. marinoi* cells (Fig. III-8). Compared to DHR123 the detection of ROS was less significant, but an increase relative to PUA concentration was still evident (Fig. III-8). The highest increase was elicited by 20  $\mu\text{M}$  OCTA 1 hour after exposure ( $1.5 \pm 0.1$  times the control, Fig. III-8). Additionally, differently from ROS, CM-H<sub>2</sub>DCFDA-derived green fluorescence exhibited a plateau 20 min after exposure, likely to indicate that the dye was being trapped inside the cells and not leaking out.

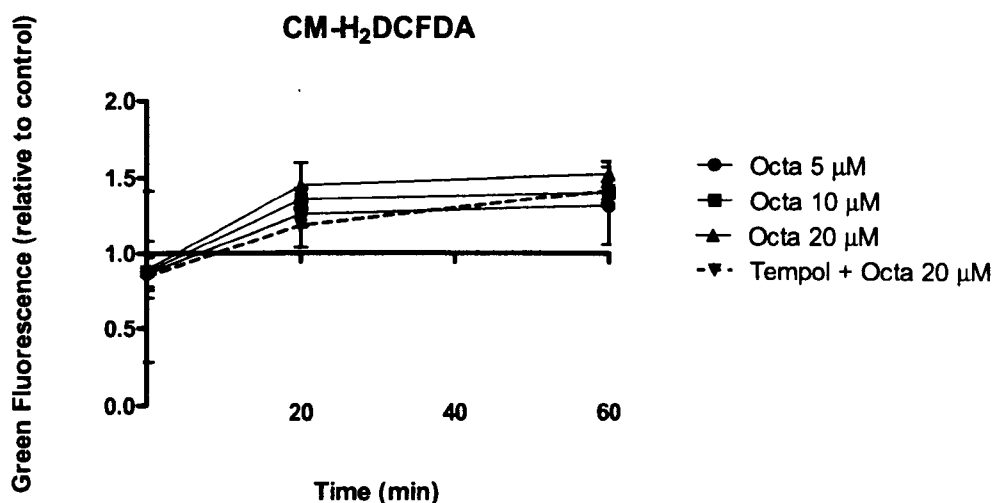


Figure III- 8: ROS production in *S. marinoi* exposed to different concentrations of OCTA. Data are expressed in terms of ROS-CM-H<sub>2</sub>DCFDA derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates. Tempol represents a ROS scavenger, inoculated with the highest concentration of PUA tested.

#### 3.2.3.4 Effect of PUAs on growth, carotenoid pigments and photosynthetic efficiency

*S. marinoi* cultures were exposed to different OCTA concentrations over 48 hours. Cell counts at 24h after exposure (Fig. III-9 a) showed that at the lowest OCTA concentration (5  $\mu$ M) the growth rate was close to that of the control ( $0.42 \pm 0.08 \text{ day}^{-1}$  and  $0.64 \pm 0.07 \text{ day}^{-1}$ , respectively), whereas at 10 and 20  $\mu$ M growth rates were  $0.11 \pm 0.15 \text{ day}^{-1}$  and  $-0.29 \pm 0.08 \text{ day}^{-1}$ , respectively (Table III-2). After 48 h the growth rate at 5  $\mu$ M OCTA was  $-0.19 \pm 0.12 \text{ day}^{-1}$ , while for the control it was  $0.03 \pm 0.02 \text{ day}^{-1}$  (Table III-2), indicating that the lowest PUA concentration had a later growth-inhibiting effect as compared to the higher concentrations. At 48 h growth rates for 10  $\mu$ M and 20  $\mu$ M were  $-0.12 \pm 0.13 \text{ day}^{-1}$  and  $-0.10 \pm 0.10 \text{ day}^{-1}$ , respectively (Table III-2), suggesting that at high concentrations OCTA had a significant effect already at 24 h. After 24 h, cultures were divided into two, and half of each culture was re-suspended in fresh medium (i.e. OCTA was removed) and cell counts were recorded up to 48 h (Fig. III-9 b). After 24 h from resuspension, growth rates of cultures exposed to the lower concentrations, 5  $\mu$ M and 10  $\mu$ M, were  $0.69 \pm 0.1 \text{ day}^{-1}$  and  $0.57 \pm 0.04 \text{ day}^{-1}$ , respectively, thus comparable to that of the control ( $0.48 \pm 0.05 \text{ day}^{-1}$ ) (Table III-2). On the contrary, growth rate of cultures previously exposed to the highest OCTA concentration (20  $\mu$ M) was  $0.09 \pm 0.07 \text{ day}^{-1}$ , and recovered to  $0.47 \pm 0.11 \text{ day}^{-1}$  after 48 h. This likely indicates that after exposure to an OCTA concentration twice the EC50 (20 M), cultures experienced a lag phase before being able to recover and resume active growth after PUA removal (Fig. III-9 b).

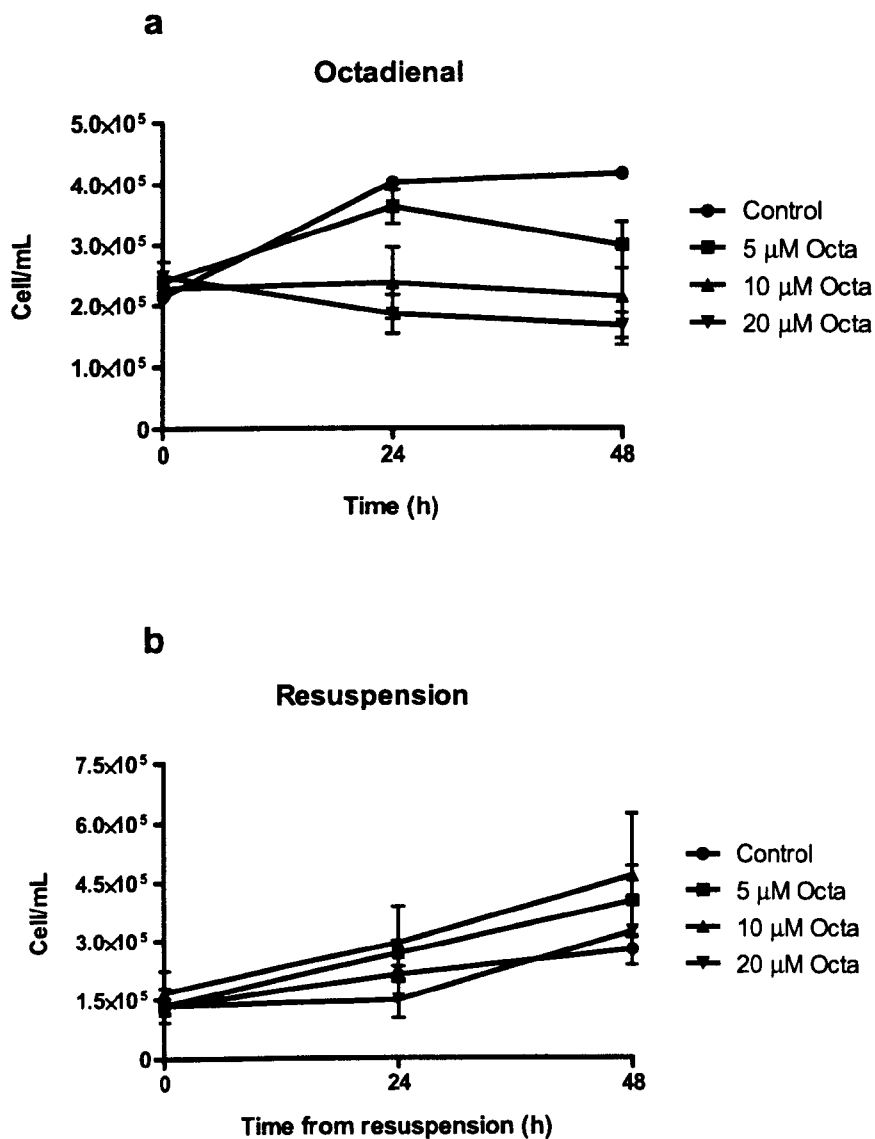


Figure III- 9: Average concentrations of *S. marinoi* cultures during exposure to different OCTA concentrations (a) and after resuspension in fresh medium, i.e. PUA removal (b). Data are means  $\pm$  SD from three biological replicates.

Table III- 2: Growth rate expressed as day<sup>-1</sup> for *S. marinoi* cultures exposed to different Octadienal concentration (OCTA) and after resuspension. Data are means  $\pm$  SD from three biological replicates.

Treatment	Time (h)	Growth rate (d <sup>-1</sup> ) OCTA	Growth rate (d <sup>-1</sup> ) Resuspension
<b>Control</b>	24	0.64 $\pm$ 0.07	0.48 $\pm$ 0.05
	48	0.03 $\pm$ 0.02	0.28 $\pm$ 0.05
	Average	0.34 $\pm$ 0.42	0.38 $\pm$ 0.14
<b>5 <math>\mu</math>M</b>	24	0.42 $\pm$ 0.08	0.69 $\pm$ 0.10
	48	-0.19 $\pm$ 0.12	0.34 $\pm$ 0.12
	Average	0.11 $\pm$ 0.44	0.55 $\pm$ 0.09
<b>10 <math>\mu</math>M</b>	24	0.11 $\pm$ 0.15	0.57 $\pm$ 0.04
	48	-0.12 $\pm$ 0.13	0.46 $\pm$ 0.08
	Average	0.00 $\pm$ 0.16	0.51 $\pm$ 0.06
<b>20 <math>\mu</math>M</b>	24	-0.29 $\pm$ 0.08	0.09 $\pm$ 0.07
	48	-0.10 $\pm$ 0.10	0.47 $\pm$ 0.11
	Average	-0.20 $\pm$ 0.14	0.48 $\pm$ 0.03

In OCTA-exposed *S. marinoi* cultures an increase in the xanthophyll Diatoxanthin (Dt) was observed at all the concentrations tested, proportional to PUA concentration. The highest increase was observed at the OCTA concentration of 20  $\mu$ M 24 hours after exposure ( $5.83 \pm 1.60$  fold increase relative to control, Fig. III-10 f). An increase in Dt over the pool of both Dt and its precursor, Diadinoxanthin (Dd), was already evident 20 min after exposure, with all the OCTA concentrations tested (Fig. III-10 a). This indicates that Dt was increasing concomitantly to a decrease of Dd which was being converted to Dt due to the activation of the xanthophyll cycle (XC) (Fig. III-10 e and c). Additionally,  $\beta$ -carotene, a xanthophyll upstream precursor, also showed a similar response to that of Dd, with a decrease already in the first 20 min after exposure (Fig. III-10 g) and a subsequent increase up to 24 h for the highest OCTA concentration tested (20  $\mu$ M), possibly indicating its protective role as an antioxidant against PUA-induced stress (Fig. III-10 h).

Dt values decreased for all the OCTA concentrations tested after 24 h from resuspension (see arrows in Fig. III-10 b, d, f, h). This was particularly evident for OCTA 20  $\mu$ M, for which there was a 43% decrease in Dt (from  $5.83 \pm 1.60$  fold increase, to  $3.30 \pm 0.12$  fold increase relative to control) (Fig. III-10 f). Concomitantly to a decrease in Dt, an increase in Dd (39% increase for 20  $\mu$ M OCTA) (Fig. III-10 d) was also evident, parallel to a decrease in the ratio of Dt over the pool of Dt and Dd (Fig. III-10 b).  $\beta$ -carotene showed a different pattern, decreasing in concentration in the first 20 min of OCTA exposure, and then increasing both during the remaining exposure time and 24 h after resuspension (Fig. III-10 g-h).

Non-photochemical-quenching (NPQ) values remained constant at a value of ca. 1 within the first 3 hours and throughout the 24 hours of exposure (Fig. III-11 c-d), suggesting that the activation of XC was not being involved in excess light energy

dissipation but rather in protecting the cells from another source of oxidative stress (i.e. PUAs). Additionally, Fv/Fm values, which measure PSII quantum efficiency, did not vary in response to OCTA exposure and remained constant at optimal growth values (ca. 0.66, Fig. III-11 a-b), indicating that the photosynthetic system was not being impaired by chemical stress.

Since no significant change was detected in Fv/Fm or NPQ over 24 h of exposure, these parameters were not measured after resuspension of cultures in fresh medium.



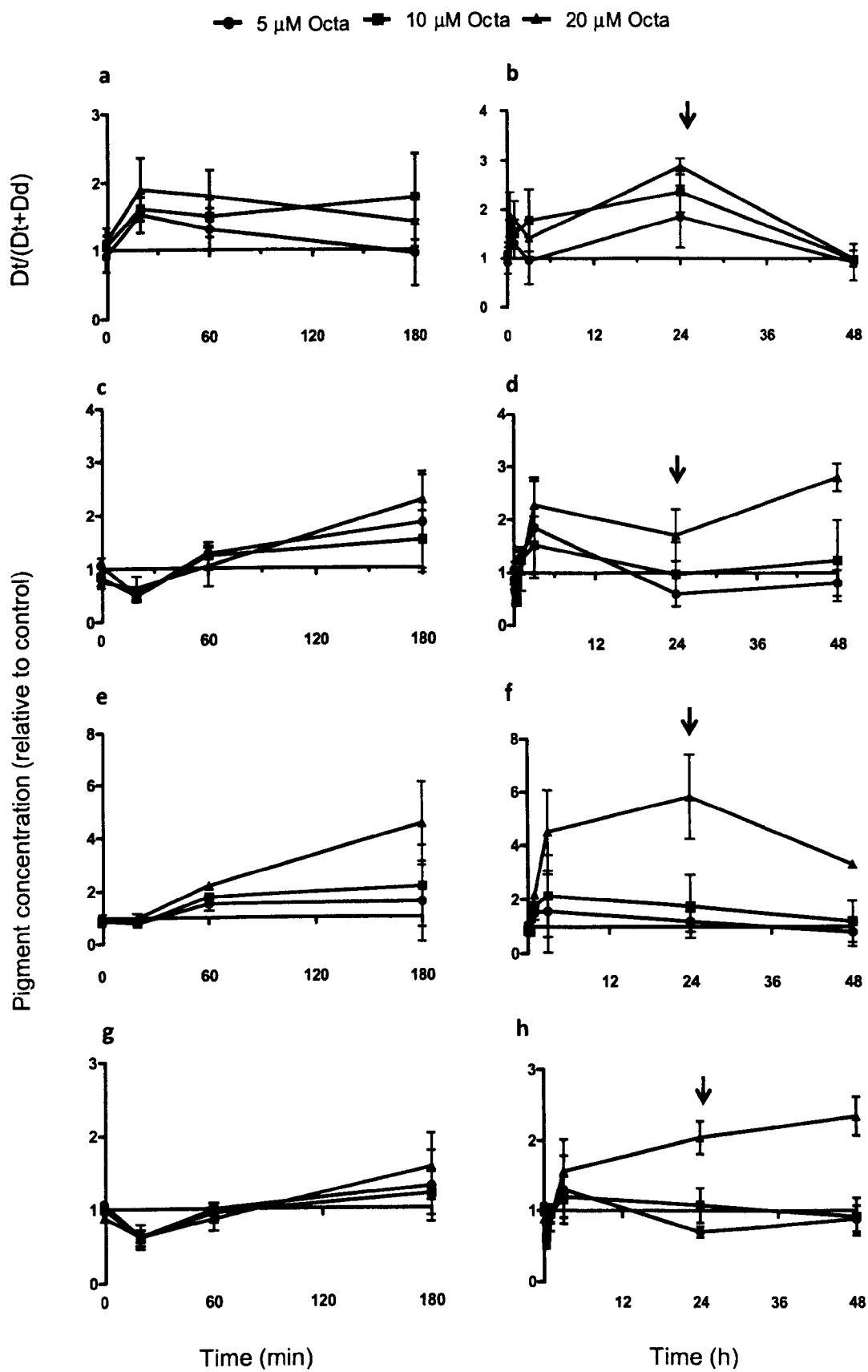


Figure III- 10: Caption on opposite page.

Figure III- 10 (a) and (b) Diatoxanthin (Dt), normalized by the sum of Dt and Diadinoxanthin (Dd) [Dt/(Dt+Dd)], relative to the control in *S. marinoi* cultures exposed to different OCTA concentrations over 180 min (a) and 48 h (b) of exposure; (c) and (d) Dd relative to the control over 180 min (c) and 48 h (d) of exposure; (e) and (f) Dt relative to the control over 180 min (e) and 48 h (f) of exposure; (g) and (h)  $\beta$ -carotene relative to the control over 180 min (g) and 48 h (h) of exposure. Arrows indicate when cultures were re-suspended in fresh medium. Data are means  $\pm$  SD from three biological replicates.

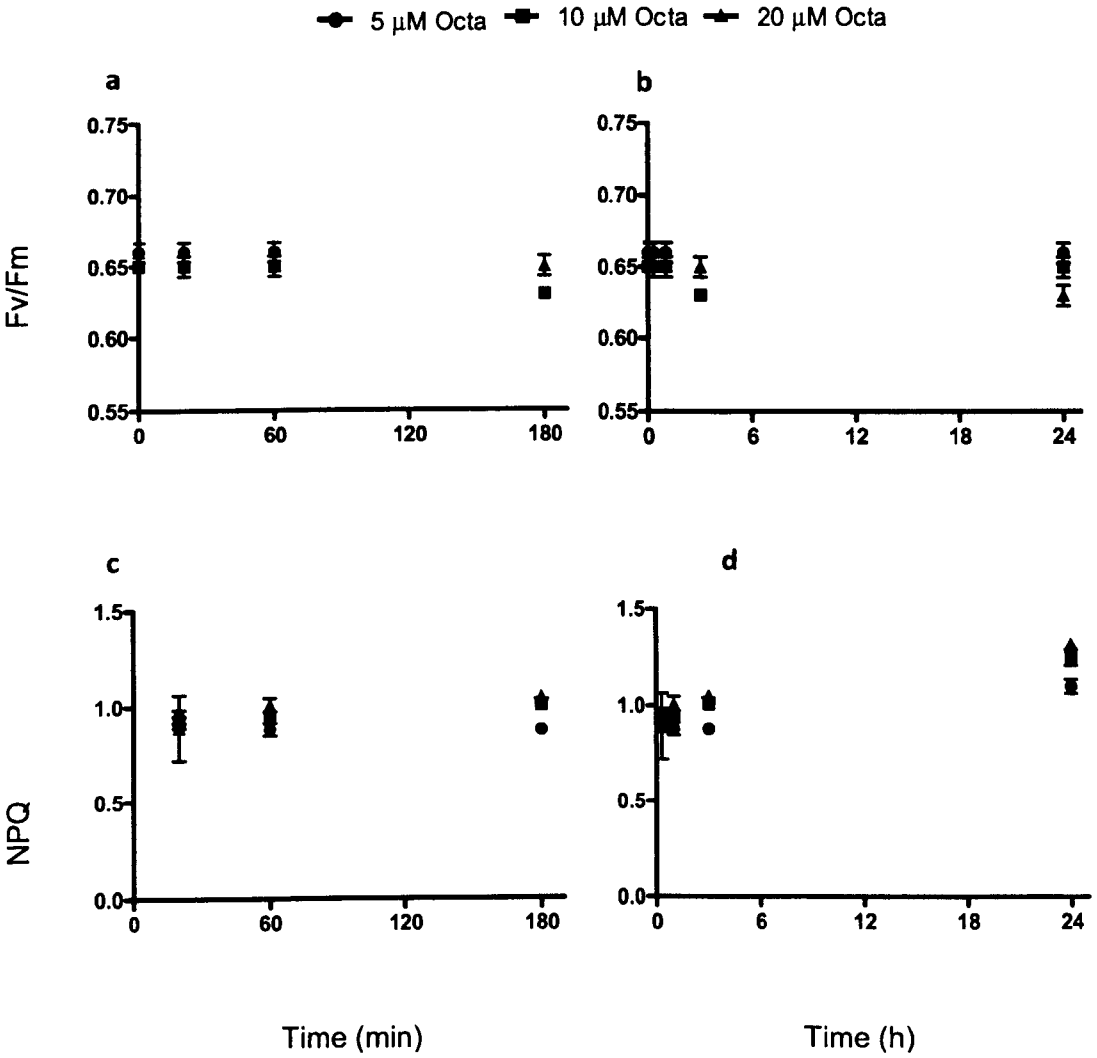


Figure III- 11: (a) and (b) Fv/Fm (maximum photochemical yield, a proxy for photosynthetic efficiency) in *S. marinoi* cultures exposed to different OCTA concentrations over 180 min (a) and 24 h (b) of exposure; (c) and (d) Non-Photochemical-Quenching (NPQ) in *S. marinoi* cultures exposed to different OCTA concentrations over 180 min (c) and 24 h (d) of exposure. Data are means  $\pm$  SD from three biological replicates.

### 3.2.4 Discussion

The observed decrease in NO upon exposure to PUA can be attributed to a shift of the stress response pathway towards the production of different reactive species. For this reason, I chose to explore the involvement of Reactive Oxygen Species (ROS) in the cell reaction to PUAs.

Results reported here show that when *S. marinoi* is exposed to PUAs other than DECA, ROS production occurs, with a peak 20 min after exposure. There appears to be a threshold for ROS production, below which no ROS are produced, represented by half the EC50 value for OCTA and the EC50 itself for HEPTA. This agrees with what has been reported by Vardi et al. (2006) in *P. tricornutum*, that a threshold-like response might exist in cells sensing a PUA-derived stress. The difference between OCTA and HEPTA is probably related to the reported stronger activity of OCTA (Ribalet et al., 2007a), due to its relatively longer C chain. ROS production has been reported in other phytoplankton species in response to different stresses (Vardi et al., 1999; Evans et al., 2006; Vardi et al., 2007; Liu et al., 2007; Jamers et al., 2009; Rioboo et al., 2011). Oxidative stress, caused by a number of different factors, including chemicals, is known to induce ROS production in algal cells (Mallick and Mohn, 2000). In the dinoflagellate *Peridinium gatunense* a thiol protease excreted by ageing cells was able to induce ROS production and concomitant cell death at the population level (Vardi et al., 2007). Additionally, the cyanobacterial toxin nodularin has been found to induce an increase in oxidative stress in the red macroalga *Furcellaria lumbricalis* (Pflugmacher et al., 2010) and different allelochemicals produced by submerged freshwater macrophytes were reported to increase ROS production in both green algae and cyanobacteria (Wang J. et al., 2011).

Surprisingly, DECA does not elicit ROS production in *S. marinoi*. This implies that ROS are involved in a response not related to simple PUA toxicity and that such response is indeed specific for PUAs that are produced by the cell itself. It should be noted that *S. marinoi* has been previously reported to exhibit a differential response in growth rate when exposed to OCTA and HEPTA as compared to DECA (Ribalet et al., 2007a), suggesting for a specificity towards the PUAs that it produces. It is therefore possible that ROS have a similar role to NO in *P. tricornutum* (Vardi et al., 2006), and that ROS are involved in the intra-population stress signalling pathway in *S. marinoi*. The ROS downstream response is likely to activate genes involved in either alternate signalling pathways or cell death cascade, depending on the PUA concentration. In the congeneric *S. costatum* (now *S. tropicum*) a cell death-specific gene (*ScDSP*) is thought to be involved in the cascade leading to cell death by apoptosis (Chung et al., 2005). Expression of this gene is enhanced by a NO-donor (Chung et al., 2008) and even stronger by hydrogen peroxide (Chung, pers. comm.). This suggests that also ROS may have an important role in the molecular cascade following PUA exposure if an apoptotic-type of cell death is hypothesized as a response, and this might have strong implications for population dynamics, as instance, during diatom blooms, when cell lysis increases and PUA are released.

In these experiments two different fluorescent ROS-sensitive dyes were used, DHR123 and CM-H<sub>2</sub>DCFDA. DHR123 has been reported to be a ROS sensitive dye that can react with a number of different species, including hydrogen peroxide, hypochlorous acid and peroxynitrite (Ischiropoulos et al., 1999). In particular, peroxynitrite is a very strong RNS, formed by the reaction of NO and superoxide anion. It is therefore possible that the green fluorescence detected in *S. marinoi* upon exposure to PUAs using DHR123 is also due to peroxynitrite formation and this could explain the observed parallel

decrease in NO. This might be also supported by the strong effect of the ROS scavenger used in our experiments (Tempol), which is considered to be a general purpose redox cycling agent (Wilcox, 2011), but it is also known to attenuate peroxynitrite (Khattab, 2006). Similarly, the presence of other ROS such as  $H_2O_2$ , which is detected by DHR123 and CM- $H_2$ DCFDA, cannot be excluded. Dichlorodihydrofluorescein diacetate probes have been reported to be indicators of intracellular  $H_2O_2$  as well as other ROS (e.g. the hydroxyl radical) and RNS (e.g. peroxynitrite). The main difference of CM- $H_2$ DCFDA with respect to DHR123 is that being a chloromethyl derivative it exhibits much better retention in live cells (West et al., 2011). ROS production assessed with the fluorescent ROS-sensitive dye CM- $H_2$ DCFDA also confirmed an increase in ROS production in *S. marinoi* exposed to OCTA (Fig. III-8). These data reinforce the findings obtained with DHR123, indicating that after 20 min maximal ROS concentration in response to PUAs was reached. Also with CM- $H_2$ DCFDA the use of the ROS scavenger Tempol induced a lower ROS-derived green fluorescence compared to treated samples (Fig. III-8).

The PUA concentrations used were half the EC<sub>50</sub>, exactly the EC<sub>50</sub> and twice the EC<sub>50</sub> values (Ribalet et al., 2007a), and they also were the concentrations at which a significant increase in ROS production was observed (5  $\mu$ M, 10  $\mu$ M and 20  $\mu$ M, see Fig. III-7). Not surprisingly, the highest concentration of OCTA tested (20  $\mu$ M) was the only one to induce a negative growth rate at 24 h (i.e.  $-0.29 \pm 0.08$ , Table III-2 ), whereas 5  $\mu$ M and 10  $\mu$ M OCTA caused an inhibition in algal growth, as already reported for different phytoplankton species exposed to PUAs (Ribalet et al., 2007a), but not a decrease in cell density. What was interesting to note was that when cells were resuspended in fresh medium after 24 hours (i.e. PUA removal), cells that had been exposed to the lower concentrations (5  $\mu$ M and 10  $\mu$ M OCTA) were able to start active growth already in the first 24 h (Fig. III-9 b) and exhibited a growth rate higher than the

control (Table III-2). This might be explained by the fact that the growth rate of cells previously exposed to low concentrations of OCTA had been inhibited, but cells maintained their ability to divide and grow (e.g. not impaired photosynthetic efficiency, see Fig. III-10 a-b). Therefore, when optimal growing conditions became available (i.e. PUA removal), cultures were able to recover at a faster growth rate compared to that of the control. Contrarily, cells exposed to OCTA 20  $\mu\text{M}$  experienced a lag growth phase within the first 24 h after PUA removal (Fig. III-9 b), while at 48 h cultures were completely able to recover and start active growth comparably to cells exposed to the lower OCTA concentrations (Table III-2). In the marine diatom *Thalassiosira weissflogii* DECA was found to inhibit cell growth in a time and dose-dependent manner, blocking cells in the G1 phase and causing irreversible effects after 24 h of exposure (Casotti et al., 2005). It should be noted that, differently from *S. marinoi*, *T. weissflogii* has not been reported to produce PUAs, (Wichard et al., 2005a), which might explain the observed difference. In order test this hypothesis, future research should test the effect of DECA exposure on *S. marinoi* in terms of xanthophylls production and recovery after PUA removal. *S. marinoi* cultures exposed to  $\mu\text{M}$  concentration of OCTA, HEPTA and OCTA and HEPTA combined together, showed a drastic decrease in cell density between one and two days after PUAs addition (Vidoudez and Pohnert, 2008). It should be noted however that the  $\mu\text{M}$  concentrations of OCTA used by Vidoudez and Pohnert (2008) were almost 3-fold higher compared to the ones used in my experiments (20  $\mu\text{M}$  vs. 62  $\mu\text{M}$ ). On the contrary, PUAs concentrations in the range of those found to be released in the medium by intact cells (i.e. nM concentrations) were not found to induce any significance change in growth in comparison with the controls (Vidoudez and Pohnert, 2008). OCTA and HEPTA nM concentrations were found to accelerate death only in the late stationary phase (Vidoudez and Pohnert, 2008). Clearly, in this type of studies the

issue of relevant ecological concentrations is a very debated and key aspect to be taken into consideration. Recently, Vidoudez et al. (2011a) reported *in situ* particulate and dissolved PUA concentrations during a spring bloom of *S. marinoi* in the Adriatic Sea (Italy). These authors found that the potential particulate PUA production by the plankton community in 1 L of seawater was in the range of nM concentrations. Despite dissolved HEPTA and OCTA concentrations were only 0.5% and 1% of the particulate PUAs where *S. marinoi* was found to be dominant, this might be explained by significant dilution effects in the moving water bodies (Vidoudez et al., 2011a). Additionally, it must be taken into consideration that when PUAs are measured at a given time, the turnover rate of these highly reactive compounds is not considered, and therefore the values underestimate the actual release rate of these molecules (Vidoudez et al., 2011a). In fact, PUA production is known to be a continuous process, which is reinitiated when the substrate is removed from the enzyme (Fontana et al., 2007b), and this might allow the maintenance of high local concentrations at sea (Ribalet et al., 2007a). Another important aspect is represented by the variability which has been reported to occur in PUAs as well as other oxylipins and other exudates produced by *S. marinoi*, in terms of seasonality, growth phase and different years during which clones were isolated (Gerecht et al., 2011; Taylor et al., 2009; Barofsky et al., 2009). This should also be an important point to be considered when addressing potential responses of PUA-producing diatoms since the evolution of different strategies both in terms of signalling or resistance based on the different nature of these compounds might be of high relevance.

It has already been reported that in the marine diatom *Thalassiosira weissflogii* PUAs are able to induce an increase in xanthophylls, for which an antioxidant role has been hypothesized (Casotti et al., 2005). In photosynthetic organisms an increase in Dt is usually associated with light stress (high light), which induces the enzymatic conversion

of Dd into Dt through the process known as non-photochemical-quenching (NPQ) responsible for excess light energy dissipation (Brunet and Lavaud, 2010, and references therein). In my experiments light remained constant and NPQ values did not show any significant variation, suggesting that in PUA-exposed *S. marinoi* cultures the activation of the XC was providing protection against a different oxidative stress factor (i.e. PUA exposure). It has been previously suggested that carotenoid xanthophylls can have a role as antioxidants during a state of oxidative stress not necessarily driven by light (Dimier et al., 2009; Schumann et al., 2007; Lavaud, 2007). Additionally, xanthophyll biosynthesis has been associated to the influence of different environmental factors, such as nutrient concentration (Staehr et al., 2002; van de Poll et al., 2005; van Leeuwe et al., 2005). An increase of (Dt+Dd)/chl*a* in response to nutrient starvation has been previously reported (Geider et al., 1993), whereas inhibition of epoxidation of Dt in diatoms due to cadmium exposure has been observed (Bertrand et al., 2001). Interestingly, Llewellyn et al. (2007) provided the first evidence of the activation of the XC in response to viral infection in *Emiliana huxley* cultures, which correlated to a decrease in photosynthetic efficiency (PE). PE has also been previously shown to be negatively affected by copper exposure (Barros et al., 2005) and viral infection (Evans et al., 2006), in parallel with an increase in ROS production. Contrarily, in my experiments, OCTA-exposed *S. marinoi* cultures did not show any significant change in the photosynthetic efficiency with all the OCTA concentration tested. This indicates that the photosynthetic apparatus was not being impaired by the chemical stressor (i.e. PUA), and suggested that *S. marinoi* cells could maintain their capacity to photosynthesize as demonstrated by the finding that all cultures were able to recover from PUA stress and continue active growth when resuspended in fresh medium, 24 h after exposure. Additionally, our data showed a different pattern of Dt increase in OCTA-exposed *S. marinoi* cultures depending on OCTA concentration.



For the lower concentrations (5  $\mu\text{M}$  and 10  $\mu\text{M}$ ) there was a gradual increase of Dt starting from 1 h after exposure, in parallel with a decrease of Dd, which was likely being converted into Dt in the first 20 min (Fig. III-10 c-f). Instead, for the highest concentration of 20  $\mu\text{M}$  OCTA, for which a negative growth rate was observed at 24 h ( $-0.29 \pm 0.08 \text{ day}^{-1}$ , Table III-2), Dd did not show any significant decrease in the first 20 min, whereas Dt significantly increased between 20 min and 1 h, by ca. 2-fold (Fig III-10 c and e). This indicates that when the chemical stress was too strong an immediate activation of the Dd-Dt cycle, i.e. de-novo synthesis of both Dd and Dt, might have occurred as a stronger way of protection. A peak in Dt content for 20  $\mu\text{M}$  OCTA treated samples was observed at 24 h (Fig. III-10 f). However, when looking at the ratio of Dt variation over the pool of both Dt and Dd, it was possible to observe an increase already after 20 min for all the OCTA treatments (Fig. III-10 a). This correlates with a decrease of Dd for 5  $\mu\text{M}$  and 10  $\mu\text{M}$  and with an increase of Dt for 20  $\mu\text{M}$  at 20 min (Fig. III-10 b and c). After resuspension (see arrows in Fig. III-10 b, d, f, h) Dt values decreased while Dd increased for all the OCTA concentrations tested (Fig. III-10 b). This indicates that at all OCTA concentrations, Dt production through the XC had been activated as a short-term response in order to cope with some level of oxidative stress likely to be induced by PUA exposure. When the chemical stress (i.e. PUA) was removed, cells were able to restore basal levels of Dt over the pool of both Dt and Dd (Fig. III-10 b).

$\beta$ -carotene showed instead a different pattern, decreasing in concentration during the first 20 min of exposure with all the OCTA concentrations tested and then increasing starting from 3 h up to 24 h, as well as after resuspension, only at the highest OCTA concentration (20  $\mu\text{M}$ ) (Fig. III-10 g-h). This is likely to be dependent on its dual role as an upstream xanthophyll precursor (Lohr and Wilhelm, 1999) as well as a general antioxidant molecule (Sigaud-Kutner et al., 2002).  $\beta$ -carotene has been previously

suggested to have antioxidant functions in marine phytoplankton species in response to viral infection (Llewellyn et al., 2007), iron limitation (Riseman and DiTullio, 2004), heavy metals (Okamoto et al., 2001) and also during the senescent phase of growth (Sigaud-Kutner et al., 2002). In my experiments  $\beta$ -carotene showed an interesting pattern with the highest concentration of OCTA tested (20  $\mu$ M). In fact, after an initial decrease in the first 20 min, it increased again after 3 h and continued to increase up to 24 h and also after cells were resuspended in fresh medium. This might indicate that  $\beta$ -carotene was involved in a long-term protection when oxidative stress was more severe, as opposed to the activation of the XC which occurred at a shorter time scale within the first 20 min and then went back to normal levels after PUA removal. Besides its potential involvement in a longer-term protection, it could also be possible that a later  $\beta$ -carotene synthesis might depend on its less strong antioxidant capacity with respect to Dt. Additionally, it has also been hypothesized that an increase in  $\beta$ -carotene synthesis might be due to a positive feedback type of mechanism following the activation of the XC (Dimier et al., 2009), which in the case of my experiments occurred only at the highest OCTA concentration, when the oxidative stress was more severe and required a stronger antioxidant cellular response. In fact,  $\beta$ -carotene content remained fairly constant with the lower OCTA concentrations, further suggesting a major role against oxidative stress only at the highest OCTA concentration. This also correlates well with the cell counts at 24 h, according to which the growth rate for the 5  $\mu$ M OCTA-treated cells was  $0.42 \pm 0.08 \text{ day}^{-1}$ , for 10  $\mu$ M was  $0.11 \pm 0.15 \text{ day}^{-1}$  and for 20  $\mu$ M was  $-0.29 \pm 0.08 \text{ day}^{-1}$ , indicating a more severe stress occurring at 20  $\mu$ M OCTA.

Altogether, my data indicate that at all the OCTA concentrations tested protection against oxidative stress was taking place in *S. marinoi* cells by means of XC activation (for a summary see Table III-3). When the stress was more pronounced (20  $\mu$ M OCTA),

which correlated with a negative growth rate at 24 h, the cellular response to oxidative stress resulted to be faster and more efficient to cope with the ROS levels produced (Fig. III-7 b). Such state of oxidative stress and consequent XC activation was not dependent on light as this was kept constant. The fact that neither NPQ or photosynthetic efficiency showed any variation within the first 24 h of exposure to PUAs demonstrates that the activation of the XC was not involved in excess light energy dissipation but that it was rather directed towards the protection of the cells against PUA-derived oxidative stress. This might be related to protection of cellular membranes, as already suggested for the xanthophyll zeaxanthin in higher plants (Havaux and Niyogi, 1999). Indeed, zeaxanthin has the same role of photoprotection as Dt, in the XC of higher plants (Demmig-Adams, 1990). On the other hand, these data also suggest that the cells were able to maintain their capacity to photosynthesize since the photosynthetic system was not being impaired, also considering that after 24 h of exposure *S. marinoi* cells were able to recover from PUA stress. Considering that in terms of ROS production an increase in ROS proportional to aldehyde concentration was observed at all the concentrations tested (Fig. III-7), it is possible to speculate that protection against oxidative stress through the activation of the XC occurred at all the concentrations at which ROS production has been observed (5  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M, Fig III-7). So it is hypothesized that an increase in Dt production might help cells in coping with the oxidative stress caused by PUA exposure. The carotenoid astaxanthin has been previously shown to play a key role in protecting *Haematococcus pluvialis* cells from oxidative stress caused by paraquat exposure (Rioboo et al., 2011). Additionally, a ROS-regulated production of carotenoid in the yeast *Phaffia rhodozyma* (Schroeder and Johnson, 1993) and in the green alga *Dunaliella bardawil* (Shaish et al., 1993) has been reported. A possible future line of research should aim at directly linking ROS production to the activation of the XC, for instance by using ROS scavengers and

enzymatic inhibitors, which would allow to verify if PUA-induced xanthophyll production is affected. Additionally, provided that more molecular tools will become available for *S. marinoi*, gene knockdown of specific genes involved in ROS and/or RNS production (e.g. SOD) could be an alternative way to test the direct link between ROS and xanthophyll production in response to PUAs.

At the highest PUA concentration (i.e. 20  $\mu\text{M}$ ), cells exhibited two different types of protective response, over the short term through the XC activation and over the longer term through  $\beta$ -carotene production (Table III-3). Additionally, cultures exposed to 20  $\mu\text{M}$  OCTA were the only ones to exhibit a negative growth rate (Table III-2), possibly indicating that in this case ROS could also be involved in eliciting a cell-death type of response, possibly through a molecular cascade involving specific gene expression, as seen for NO and the gene *ScDSP* in *S. costatum* (now *S. tropicum*) (Chung et al., 2008). It is important to note that even if exposure to higher PUA concentration induced a negative growth rate, cells were not affected in their photosynthetic efficiency, and in fact they were able to recover after PUA removal (Fig. III-9 b). Probably this differential response might also be present at sea during a bloom condition, when local PUA concentrations are expected to be high (Vidoudez et al., 2011a). This would imply that the type of response might depend both on the concentration to which cells are exposed to and possibly also the time of exposure. In fact, in my experiments cells were exposed to PUAs for up to 24 h. However, *in situ* conditions during a bloom are likely to be prolonged in time (Vidoudez et al, 2011a). It is known that PUAs are released upon cell lysis as well as during the late stationary phase of growth by intact *S. marinoi* cells (Vidoudez and Pohnert, 2008). This is of particular significance in terms of PUA dynamics at sea during the termination of a bloom, when lysis rate is expected to be high. In fact it indicates that the cellular response of a PUA-producing diatom to PUAs could

be finely modulated by and highly dependent on the time and dose of exposure to PUAs themselves, both in terms of ROS production and antioxidant defence. What could be the case during the termination of a bloom is that after PUAs are released, depending on both the PUA concentration and the time of exposure, *S. marinoi* cells could either experience ROS-mediated protection and/or apoptotic cell death, as seen for DECA-exposed *T. weissflogii* (Casotti et al., 2005). For those cells where a protective type of response rather than cell death is elicited, there will likely be a slowing down of the growth rate but constant photosynthetic ability. This is expected to allow cells to quickly recover when the chemical stressor will no longer be present in the environment, allowing cells to restore the original population size. An interesting aspect is that in the green alga *Haematococcus pluvialis* the biosynthesis of the carotenoid astaxanthin is enhanced in cysts by oxidative stress induced by addition of reactive oxygen species (Kobayashi et al., 1993). Additionally, a survival strategy involving cyst formation has been reported for the dinoflagellate *Heterocapsa circularisquama* in the presence of another dinoflagellate and different diatoms (Uchida et al., 1999). More recently, the dinoflagellate *Scrippsiella trochoidea* was found to form temporary cysts in response to exposure of low concentrations of allelochemicals released by three toxic microalgae, whereas higher concentrations induced cell death (Fistarol et al., 2004). It is intriguing to speculate that something similar might also occur in *S. marinoi* during the end of a bloom at sea, where PUAs might act as infochemicals within the same diatom population by inducing cyst formation. The fact that in the experiments reported here no cyst formation was detected might be dependent on the relatively short time scale that has been investigated. Interestingly, resting stages of the congeneric *S. costatum* were found to survive for several decades in the sediments of a Swedish fjord (McQuoid et al., 2002). This is of particular relevance from an evolutionary perspective. In fact it is believed that chemical

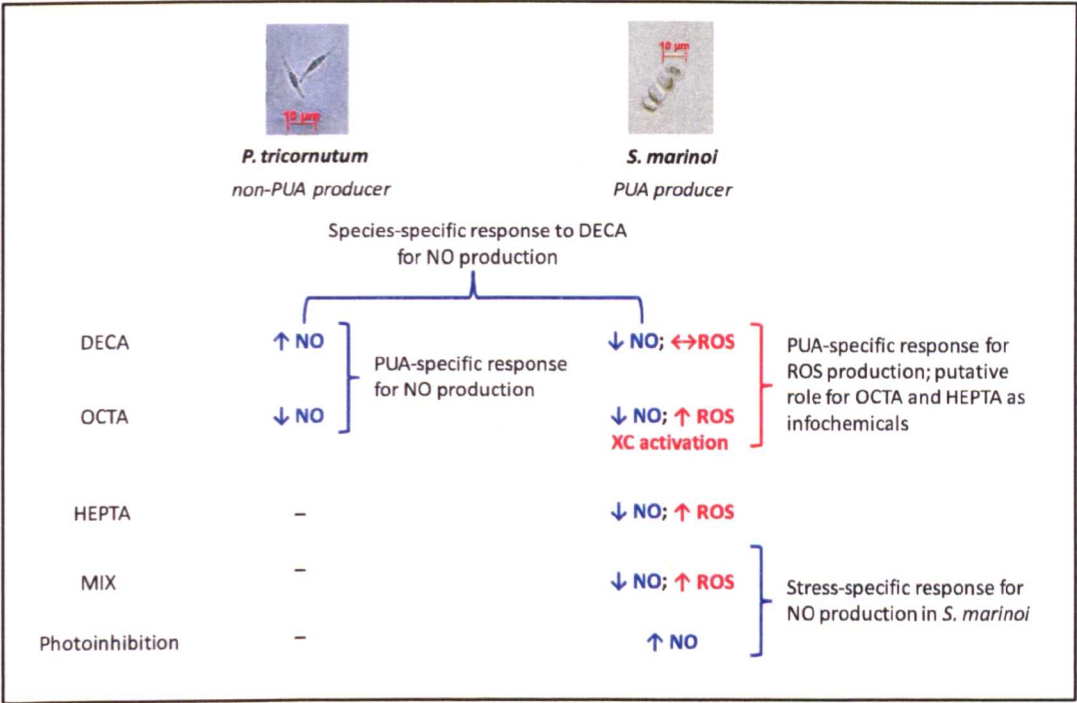
mediated interactions might have driven the evolution of certain organisms by selecting those individuals that had the ability to either resist to, exploit or avoid external metabolites from neighbouring cells (Lucas, 1947). It has also been claimed that this could only be expected if chemical-mediated interactions were sporadic, as occurs during bloom events (Lewis, 1986).

Recently, it has also been reported that sex pheromones present in cell filtrates were able to induce sexualisation in the pennate diatom *Pseudostaurosira trainorii* (Sato et al., 2011), which might add additional relevance to the role of released metabolites as infochemicals within the same diatom population, for instance inducing sexual reproduction under particular environmental conditions. It should also be noted that in the green alga *Volvox carteri* sex has been demonstrated to occur in response to high levels of ROS, likely to be responsible for the activation of sex genes (Nedelcu et al., 2004; Nedelcu, 2005). It is then possible to speculate that in certain microalgal species a link might exist between the release of and exposure to chemical compounds (e.g. PUAs), induced ROS production and activation of sex genes.

Table III- 3: Summary of the results on xanthophyll production in OCTA-exposed *S. marinoi* cells. = indicates that the difference between the treatment value and the control value is  $\leq$  or  $\geq$  20%; - indicates that the decrease of the treatment value with respect to the control value is  $>$  20%; + indicates the increase of the treatment value with respect to the control value is  $>$  20%; ++ indicates that the increase of the treatment value with respect to the control value is  $>$  100%. OCTA 2E,4E/Z octadialenal; Dd: Diadinoxanthin; Dt: Diatoxanthin.

20 min after exposure				
	Dd	Dt	Dt/(Dd+Dt)	$\beta$ -carotene
5 $\mu$ M OCTA	-	=	+	-
10 $\mu$ M OCTA	-	=	+	-
20 $\mu$ M OCTA	-	=	+	-
24 h after exposure				
	Dd	Dt	Dt/(Dd+Dt)	$\beta$ -carotene
5 $\mu$ M OCTA	-	=	+	-
10 $\mu$ M OCTA	=	+	++	=
20 $\mu$ M OCTA	+	++	++	++
24 h after resuspension (following 24 h of exposure)				
	Dd	Dt	Dt/(Dd+Dt)	$\beta$ -carotene
5 $\mu$ M OCTA	=	=	=	=
10 $\mu$ M OCTA	=	=	=	=
20 $\mu$ M OCTA	++	++	=	++

Overall, data presented in Chapter 3 suggest that there is a different pathway in response to the PUA to which a diatom is exposed to (Fig. III-12).



PUA: polyunsaturated aldehydes; NO: nitric oxide; ROS: reactive oxygen species; XC: xanthophyll cycle; DECA: 2E,4E/Z-decadialenal; OCTA: 2E,4E/Z-octadialenal; HEPTA: 2E,4E/Z-heptadialenal; MIX: combination of OCTA and HEPTA. (↑) increase; (↓) decrease; (↔) no change.

Figure III- 12: Schematic representation summarising the results of Chapter 3 and their interpretation



This is evident for DECA, which elicits opposite responses when compared to OCTA and HEPTA, for instance, in NO production in *P. tricornutum* or in ROS production in *S. marinoi*. *S. marinoi* is a known producer of PUAs, and in particular of OCTA and HEPTA (Fig. III-12). These are also the most common PUAs represented in marine diatoms and also dissolved in the sea after a bloom (Vidoudez et al., 2011a). By contrast, *P. tricornutum* does not produce any PUAs (Wichard et al., 2005a). From an ecological point of view this is particularly interesting because it suggests that diatom species perceive and are able to discriminate the PUAs they are exposed to, based on their previous experience. It is then possible to speculate that *S. marinoi* recognizes OCTA and HEPTA as intracellular signalling molecules, and uses them as infochemicals, while *P. tricornutum* reacts to PUAs as external stimuli (probably allelochemicals) (Leflaive and Ten-Hage, 2009). These different responses probably underlie the ecological significance and behaviour of these two marine diatoms in the natural environment. *S. marinoi* is a widespread and bloom-forming marine diatom (Kooistra et al., 2008), whereas *P. tricornutum* is not very well represented in the natural environment and it has not been reported to form blooms in nature (De Martino et al., 2007).

In conclusion, it is suggested that the interactions between chemical signals and reactive pathways underlie the functional diversity of species and their ability to cope with the environment. Indeed, the physiological responses to stimuli and biological interactions are intertwined and can shape the ecosystems in a dynamical way, determining the ecological success of a species and its role.

## CHAPTER 4

# Pathways of Reactive Nitrogen Species (RNS) and Reactive Oxygen Species (ROS) production in *S. marinoi* in response to PUAs

### 4.1 Introduction

Reactive oxygen species (ROS) are a group of molecules with individually distinct chemical and biological properties (Nathan, 2003). ROS are normal by-products of oxidative metabolism and are known to act as important signalling molecules (Vranova et al., 2002), but are toxic and harmful when present in high concentrations. In particular, ROS can oxidize lipids, proteins and nucleic acids, damaging cell structure and causing organelle dysfunction and mutagenesis (Halliwell and Gutteridge, 1999). The term ROS generally indicates all oxidation and excitation states of oxygen ( $O_2$ ), which include the superoxide anion ( $O_2^-$ ), the singlet oxygen ( $^1O_2$ ), hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radical ( $^{\bullet}OH$ ) (Barros et al., 2005). Production of ROS represents a particularly severe hazard to photosynthetic organisms, since a common biological source of  $O_2^-$  is the singlet-electron reduction of molecular oxygen by electron transport chains. Due to the intense electron flux in an elevated oxygen and metal ion microenvironment, the chloroplasts and mitochondria of photosynthetic organisms are cell compartments highly susceptible to oxidative injury (Halliwell and Gutteridge, 1999). Chloroplasts, because of their photosynthetic nature, are hyperoxic, produce ROS and are particularly exposed to

oxidative stress (Lesser, 2006). Indeed,  $^1\text{O}_2$ ,  $\text{O}_2^-$  and  $^{\bullet}\text{OH}$  are produced in the photosystem II (PS II) reaction centre (Macpherson et al., 1993; Liu et al., 2004).  $\text{O}_2^-$  can also be generated by oxygen photochemical reduction in photosystem I (PSI) through the Mehler reaction (Asada and Takahashi, 1987; Asada, 1999).  $\text{O}_2^-$  can then diffuse into the stroma, where it is converted into  $\text{O}_2$  and  $\text{H}_2\text{O}_2$ ; the latter can then react with  $\text{Fe}^{2+}$  or  $\text{Cu}^{2+}$  ions through the Fenton reaction producing the powerful oxidant  $^{\bullet}\text{OH}$ , which is the most reactive oxygen radical (Fig. IV-1) (Takeda et al., 1995). The production of  $\text{O}_2^-$  has been reported to increase under particular conditions, for instance exposure to xenobiotics or pollutants, high light, UV radiation and thermal stress (Asada, 1994; Asada and Takahashi, 1987). If such elevated production cannot be counteracted by appropriate antioxidant defences, it will in turn cause damage to both PSII and the carbon fixation process (Lesser, 2006).

Mitochondria represent an important site of  $\text{O}_2^-$  generation, and in particular at the level of the NADH dehydrogenase at complex I and at the interface between ubiquinone and complex II (Brookes, 2005).  $\text{O}_2^-$  can be converted into  $\text{H}_2\text{O}_2$  by spontaneous dismutation or by the enzyme superoxide dismutase (SOD) (Fig. IV-1) (Brookes, 2005). Additionally,  $\text{O}_2^-$  can be produced by NADPH-dependent electron transport involving cytochrome P-450 in the endoplasmic reticulum of animals, plants and some bacteria (Halliwell and Gutteridge, 1999). Finally, peroxisomes, which are single membrane-bound subcellular organelles, also represent important sites of oxidative metabolism in both animals and plants (Singh, 1996; Corpas et al., 2001).

The main precursor of the broadly termed reactive nitrogen species (RNS) is nitric oxide (NO), which is a small, uncharged molecule which freely diffuses throughout biological membranes (Stamler et al., 1992). After production, NO can be converted into different other redox activated forms, including peroxynitrite ( $\text{ONOO}^-$ ), nitrous oxide

(N<sub>2</sub>O), nitrosonium cation (NO<sup>+</sup>), and nitroxyl anion (NO<sup>-</sup>) (Stamler et al., 1992). Inside the cytosol and specific compartments of plant and algal cells, e.g. chloroplasts and peroxisomes, NO can react with O<sub>2</sub><sup>-</sup> radicals to form the powerful ONOO<sup>-</sup> (Fig. IV-1) (Barros et al., 2005). This reaction has been reported to be almost three times faster than the SOD-catalyzed dismutation of O<sub>2</sub><sup>-</sup> radicals (Augusto et al., 2002). In higher plants it has also been reported that the same stress condition can induce the production of both NO and H<sub>2</sub>O<sub>2</sub> and that together they mediate the downstream cellular responses to oxidative/nitrosative stress (Neill et al., 2002b).

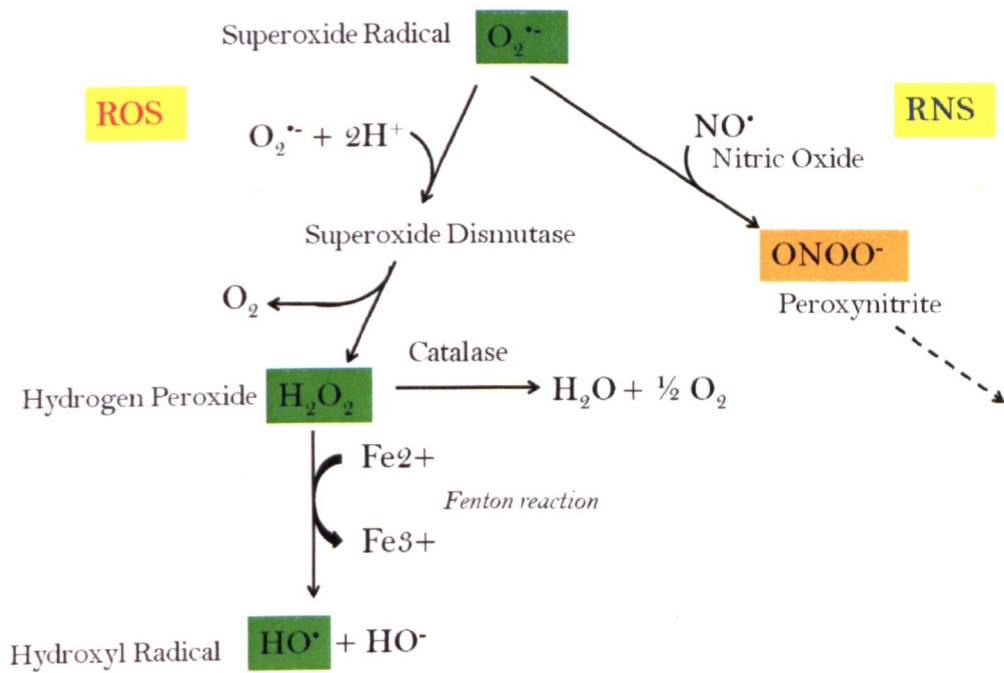


Figure IV- 1: Biochemical reactions involved in the generation of reactive oxygen (ROS) and nitrogen (RNS) species and the interplay between these two reactions. Modified from Koppenol (1998).

The relative levels of molecular and enzymatic antioxidants inside plant and algal organelles, determine the regulation and interplay between ROS and RNS inside the cell (Barros et al., 2005). Such modulation can possibly lead to a highly co-regulated communication between different cellular compartments both under physiological and stress conditions (Corpas et al., 2001). As a consequence, the biochemical behaviour of ROS and RNS in biological systems should be considered in parallel, since these reactive species interact in a number of different reactions and share important physiological functions (Barros et al., 2005).

In order to counteract the potential damaging effects of both ROS and RNS, organisms have developed different protective mechanisms through the production of different compounds. These can be divided into enzymatic catalysts of high molecular weight and low molecular weight compounds (Barros et al., 2005). The first group is represented by enzymes such as catalases (CAT), peroxidases (PX) and superoxide dismutases (SOD) (Djordjevic, 2004). The second type involves non-enzymatic, low-molecular weight compounds, such as tocopherols (e.g. Vitamin E), ascorbic acid, uric acid, glutathione (GSH), carotenoids (Salem, 1997) and dimethylsulfoniopropionate (DMSP) (Sunda et al., 2002; McLenon and DiTullio, 2012).

SOD catalyzes the conversion of  $O_2^-$  to  $O_2$  and  $H_2O_2$ , and has been recognized as the first cellular defence against ROS and RNS (Hassan and Scandalios, 1990). In photosynthetic organisms SOD occurs as three different isoforms, which have different cellular distribution. (Asada, 1999). The Cu/Zn SOD is localized mainly in the cytosol in eukaryotes, but it can also be found in chloroplasts and peroxisomes (Halliwell and Gutteridge, 1999; Sandalio and Delrio, 1988). Instead, the Mn form of SOD is found mainly in mitochondria, while the FeSOD is localized in the chloroplast stroma (Asada, 1999). The MnSOD and FeSOD are thought to be more ancient forms, and are found

principally in prokaryotic cells and eukaryotic algae (Wolfe-Simon et al., 2005), whereas the Cu/Zn SOD isoform has been reported to be present in animals, higher plants and in the green alga *Spirogyra* sp. (Wolfe-Simon et al., 2005; Kanematsu and Asada, 1989). Additionally, a nickel form of SOD (NiSOD) was discovered in the bacterial genus *Streptomyces* (Youn et al., 1996; Barondeau et al., 2004; Wuerges et al., 2004). NiSOD has a completely different structure from all the other types of SODs, and has been suggested to be active in cyanobacteria (Palenik et al., 2003). Different types of stress can induce an increase in SOD activity, such as heat stress (Rady et al., 1994), UV-B radiation (Malanga and Puntarulo, 1995), as well as heavy metals (Okamoto et al., 1996; Okamoto and Colepicolo, 1998; Collen et al., 2003) and paraquat exposure (Vartak and Bhargava, 1999). CAT is a heme-containing enzyme that catalyzes the conversion of  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$  and  $\text{O}_2$  (Fridovich, 1998; Halliwell and Gutteridge, 1999). Among  $\text{H}_2\text{O}_2$ -degrading enzymes, CAT is the only one that degrades  $\text{H}_2\text{O}_2$  without consuming cellular reducing equivalents (Mallick and Mohn, 2000). An increase in catalase activity has been reported in the photosynthetic prokaryote *Prochloron* sp. in response to light stress (Lesser and Stochaj, 1990), to heat stress in the cyanobacterium *Synechocystis* (Rady et al., 1994), and to UV-B radiation in the green alga *C. vulgaris* and in the cyanobacterium *A. doliolum* (Malanga et al., 1999; Mallick and Rai, 1999). Peroxidases (PXs), similarly to CAT, catalyze the reduction of  $\text{H}_2\text{O}_2$  to  $\text{H}_2\text{O}$ , but they require a source of electrons that subsequently becomes oxidized (Halliwell and Gutteridge, 1999). Ascorbate peroxidase (APX) is a heme-containing enzyme that uses ascorbate as the electron donor, and is mainly active in the stroma and on the thylakoids of chloroplasts (Asada and Takahashi, 1987). Glutathione peroxidase (GPX) is instead a tetrameric enzyme which catalyzes the oxidation of glutathione, a low molecular weight thiol compound, with  $\text{H}_2\text{O}_2$  (Halliwell and Gutteridge, 1999). A general stimulation of these antioxidant enzymes has been

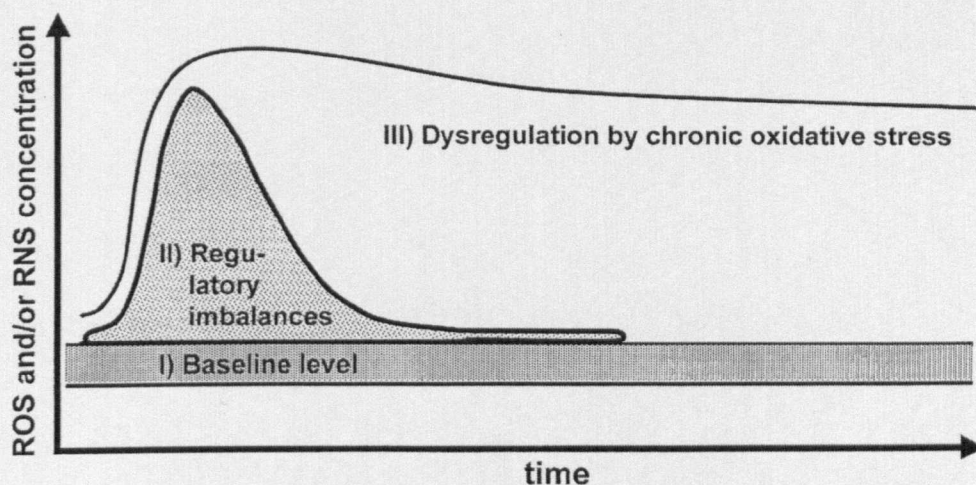
reported to occur under high irradiance (Lesser and Stochaj, 1990), water deficiencies (Smirnoff, 1993) and temperature stress (Rady et al., 1994).

Among the low-molecular weight antioxidants, carotenoids are lipid-soluble pigments that are found in bacteria, yeast, algae, plants, animal and humans (Britton et al., 1995). In all these different organisms they are known to have an important role in the protection from oxidative damage. Photoautotrophs have the ability to produce carotenoids *de novo*, while animals must assimilate these molecules through their diet (Cadenas, 1989). In photosynthetic organisms carotenoids are known to have both a light-harvesting and a protective role from oxidative stress (Frank and Cogdell, 1996; Demmig-Adams and Adams, 1993; Pinto et al., 2003). Ketocarotenoids and astaxanthin were found to increase under excessive oxidative stress in cyst cells of the green alga *Haematococcus pluvialis* (Kobayashi et al., 1997), while in the green alga *C. vulgaris*  $\beta$ -carotene was found to increase under UV-B stress (Malanga et al., 1997).  $\text{Cu}^{2+}$  exposure was also found to induce a significant increase in carotenoid content in the cyanobacterium *A. doliolum* (Mallick and Rai, 1999). Other non-enzymatic antioxidants include ascorbic acid (vitamin C), which is capable of scavenging  $\text{H}_2\text{O}_2$  as well as  $\text{O}_2^-$  and  $\text{HO}^\bullet$  (Fridovich, 1998; Asada and Takahashi, 1987). Ascorbate levels were found to increase in the freshwater *Peridinium gatunense* in response to high irradiance (Butow et al., 1997). Additionally, glutathione (GSH) is a tripeptide which represents the major low-molecular weight thiol present in cells (Ahamad, 1995). Under oxidative stress conditions, ROS are reduced by GSH with concomitant formation of the oxidized disulphide, GSSG (Halliwell and Gutteridge, 1999). GSH levels were reported to decrease in *A. doliolum* under Cd and  $\text{Cu}^{2+}$  stress (Mallick and Rai, 1999; Mallick et al., 1994). However, in *C. vulgaris*, the total thiol content was found to increase under UV-B exposure (Malanga et al., 1999). Additional low-molecular weight compounds that have



been reported to have an antioxidant function in algal cells include tocopherols (e.g. vitamin E), melatonin and mycosporine-like amino acids (MAA) (Malanga and Puntarulo, 1995; Balzer, 1996; Dunlap and Yamamoto, 1995).

When generation of ROS/RNS together with the cellular scavenging ability are constant and in equilibrium, biological systems are considered to be in a stable state. For redox signalling to occur, an imbalance of this steady-state has to take place, which can happen either due to an increase in ROS/RNS production or to a decrease in the activity of the antioxidant machinery (Dröge, 2002). If the level of ROS increase is not too high, the cellular antioxidant capacity may be adequate to restore the initial balance between ROS/RNS production and their scavenging ability (baseline level) (Fig. IV-1, condition I). This stable state condition occurring over the long term is called redox homeostasis (Dröge, 2002). However, if the level of ROS increases to an extent to which the oxidative response cannot reset the original balance, the system could on one hand still reach an equilibrium (Fig. IV-2, condition II) but with an associated higher level of ROS, which might result in changes in the redox-sensitive signalling pathways (Dröge, 2002). On the other hand, if persistent generation of high levels of ROS occurs, this might result in a chronic shift of the level of redox homeostasis (Fig. IV-2, condition III) (Dröge, 2002).



From Dröge (2002)

Figure IV- 2: Regulatory events and their imbalance depend on the magnitude and duration of the change in ROS or RNS concentration. ROS and RNS normally occur in living cells at relatively low steady-state levels. The regulated increase in superoxide ( $O_2^-$ ) or nitric oxide (NO) production leads to a temporary imbalance that is at the basis of redox regulation. However, if the production of ROS or RNS is continued, this may cause persistent changes in the signal transduction and gene expression, which can then lead to a condition of chronic oxidative stress.

Given the complexity of ROS and RNS, both in terms of pathways of production and interplay between them, it would seem obvious that they cannot be considered as single or separate molecules, but that they rather represent a broad and interconnected range of reactive species, with different biochemical and biological reactivity (Kalyanaraman et al., 2012). Additionally, both ROS and RNS are characterized by specific features that make their detection difficult, namely their short life time and the possible interference of naturally occurring antioxidants capable of scavenging these species (Gomes et al., 2006). Fluorescent probes are particularly useful because of their sensitivity and applicability in the temporal and spatial monitoring of such reactive species, especially in biological systems *in vivo* (Gomes et al., 2006; McQuade and Lippard, 2010). In this perspective, it is essential to develop methods to detect and characterize ROS and RNS as accurately as possible, and the proper use of fluorescent probes for the detection of reactive oxygen and nitrogen species is in fact a major topic of discussion (Kalyanaraman, 2011; Kalyanaraman et al., 2012; Gomes et al., 2006; Ischiropoulos et al., 1999).

Dihydrorhodamine 123 (DHR123) has been utilized for the detection of ROS in different cell types (Rothe et al., 1988; Royall and Ischiropoulos, 1993; Vardi et al., 1999; Jamers et al., 2009; Rioboo et al., 2011). However, it has also been reported that DHR123 can serve as an indicator of RNS derived from nitric oxide (NO), but not of NO itself (Ischiropoulos et al., 1999, and references therein). It has also been confirmed that neither  $O_2^-$  nor  $H_2O_2$  alone have the ability to oxidize DHR123 (Szabo et al., 1995; Kooy et al., 1994; Ischiropoulos et al., 1996; Crow, 1997). The only biological oxidants reported to be able to oxidize DHR123 are the powerful oxidants derived from the reactions of  $H_2O_2$  with peroxidases or metals, peroxynitrite ( $ONOO^-$ ), and hypochlorous acid (Ischiropoulos et al., 1999).

It has been suggested that in those cells where NO is produced, DHR123 oxidation is highly likely to be linked to a ONOO<sup>-</sup> component (Ischiropoulos et al., 1999). In terms of ONOO<sup>-</sup> involvement in DHR123 oxidation, Kooy et al. (1994) suggested that the formation of rhodamine 123 from DHR123 is the result of a direct reaction with ONOO<sup>-</sup>. On the contrary, (Jourdeuil et al., 2001) suggested that the oxidation of DHR123 by ONOO<sup>-</sup> is dependent on the free radical intermediates hydroxyl radical (HO<sup>•</sup>) and nitrogen dioxide (<sup>•</sup>NO<sub>2</sub>), which are formed through the spontaneous decomposition of ONOO<sup>-</sup> at physiological pH, thus excluding a direct reaction of DHR123 with ONOO<sup>-</sup>. Nevertheless, the exact oxidation mechanism between DHR123 and ONOO<sup>-</sup> is still a matter of debate (Gomes et al., 2006).

Oxidation of DHR123 by concomitant generation of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> requires the presence of a metal (Royall and Ischiropoulos, 1993; Kooy et al., 1994). For DHR123 oxidation by H<sub>2</sub>O<sub>2</sub>, the presence of heme proteins such as cytochrome *c* (Hempel et al., 1999) or peroxidases (McQuade and Lippard, 2010) is also necessary. It is important to note that DHR123 oxidation has been reported to be more H<sub>2</sub>O<sub>2</sub> dependent (Ischiropoulos et al., 1999 and references therein). In previous studies DHR123 was shown to be specifically responsive to H<sub>2</sub>O<sub>2</sub> (Henderson and Chappell, 1993; Qin et al., 2008; Walrand et al., 2003). DHR123 oxidation by H<sub>2</sub>O<sub>2</sub> is a slow reaction, unless catalysed by an enzyme with peroxidase activity or by peroxynitrite anion, which then results in the formation of the cationic, fluorescent, rhodamine 123 (Dikshit and Sharma, 2002).

In general, it is clear that DHR123 oxidation involves more than one oxidant; as a consequence, it has been previously suggested that the identification of the reactive species responsible for its oxidation could be obtained by the use of appropriate inhibitors and scavengers (Ischiropoulos et al., 1999). In term of scavengers, the small molecular weight scavenger uric acid has been reported to inhibit peroxynitrite-mediated DHR

oxidation (Ischiropoulos et al., 1999), while Tempol (4-Hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) is a nitroxide compound which has been reported to be a general redox cycling agent acting as a catalase (CAT)-like agent and preventing the generation of  $\cdot\text{OH}$  from  $\text{H}_2\text{O}_2$  in the presence of transition metals in the Fenton reaction (see Fig. IV- 1) (Wilcox and Pearlman, 2008). However, it has also been reported that Tempol can attenuate  $\text{ONOO}^-$  and  $\text{ONOO}^-$ -mediated effects (Khattab, 2006; Carroll et al., 2000; Bonini et al., 2001). In term of inhibition of SOD activity, it has been reported that the SOD inhibitor 2-methoxyestradiol (2ME) has the ability to inhibit SOD with consequent increase of  $\text{O}_2^-$  and decrease of  $\text{H}_2\text{O}_2$  levels (Chen et al., 2009), and that diethyldithiocarbamate (DETC) is a Cu/Zn SOD blocker which chelates and removes Cu (II) ions from the enzyme active site (Khazaei et al., 2009). In terms of NO synthesis, LNAME ( $\text{N}^G$ -Nitro-L-arginine-methyl ester . HCl) has been reported to be a general inhibitor of nitric oxide synthase (NOS), while DNAME ( $\text{N}^G$ -Nitro-D-arginine-methyl ester . HCl) is its inactive form (Rees et al., 1990). It is known that tungstate can be substitute for molybdenum (Mo) and inhibit Nitrate Reductase (NR) activity by preventing the formation of an active molybdenum cofactor, that is necessary for the catalytic activity of NR (Notton and Hewitt, 1971). Even though tungstate specificity for NR inhibition has been recently suggested to be treated with caution (Xiong et al., 2012), thanks to its simple application and common availability, tungstate has been widely used as a NR inhibitor in plant and algal NO research (Mallick et al., 1999; Freschi et al., 2010; Negi et al., 2010).

In the following experimental section, in order to better elucidate the involvement of specific ROS and RNS in the oxidative response of *S. marinoi* to PUAs, a pharmacological approach has been applied. By pharmacological approach it is intended the application of different scavengers of NO, ROS and peroxynitrite and inhibitors of

both NO synthesis and SOD activity. More specifically, cPTIO has been used as a NO scavenger, uric acid as a peroxynitrite scavenger and tempol as a ROS scavenger. In terms of inhibitors, LNAME and DNAME have been selected for NO biosynthesis, while 2ME and DETC have been chosen as SOD inhibitors.

## 4.2 Materials and Methods

### 4.2.1 Preparation of Chemicals

For ROS and RNS detection *in vivo* the fluorescent ROS-sensitive dye DHR123 was used (see Materials and Methods 3.2.2.2, Chapter 3).

For polyunsaturated aldehydes (PUAs) preparation see Materials and Methods 3.1.2.2, Chapter 3.

As scavengers, the NO scavenger carboxy-PTIO (cPTIO) (Enzo Life Sciences, Vinci, Italy), and the ROS scavenger 4-Hydroxy-TEMPO (Tempol) (Sigma-Aldrich, Milan, Italy) were used. Final working concentration were 100  $\mu$ M and 5 mM, respectively, dissolved in filtered sea water (FSW). The NO inhibitors used were L-NAME-hydrochloride (LNAME, N<sup>G</sup>-Nitro-L-arginine-methyl ester-HCl) (Enzo Life Sciences, Vinci, Italy) and sodium tungstate dehydrate (ST) (Sigma Aldrich, Milan, Italy). Final working concentrations were 1 mM and 0.5 mM, respectively, dissolved in filtered sea water (FSW). As a negative control for LNAME, its inactive form, D-NAME-hydrochloride (DNAME, N<sup>G</sup>-Nitro-D-arginine-methyl ester-HCl) (Enzo Life Sciences, Vinci, Italy) was used. Final working concentration was 1 mM. Uric Acid (Sigma-Aldrich, Milan, Italy) was used as a peroxynitrite (ONOO<sup>-</sup>) scavenger. A stock was freshly prepared in NaOH 60 mM and final working concentration was 100  $\mu$ M. The pH of the growth medium was not altered by the addition of NaOH.

As superoxide dismutase (SOD) inhibitors, two different inhibitors were used, 2-methoxyestradiol (2ME) and sodium diethyldithiocarbamate trihydrate (DETC) (both from Sigma-Aldrich, Milan, Italy). Final working concentrations were 1 mM dissolved in dimethylsulfoxide (DMSO) (Sigma-Aldrich, Milan, Italy) for 2ME, and 1 mM dissolved

in filtered sea water (FSW) for DETC. Samples were incubated for 120 min before PUAs addition

#### *4.2.2 Flow Cytometry*

For Flow Cytometry see Materials and Methods 2.1.2.3, Chapter 2.

#### *4.2.3 Statistical Analysis*

Statistical significance with respect to control values was evaluated with the Student's *t*-test using the Excel spreadsheet (Microsoft Office).



### 4.3 Results

#### 4.3.1 NO and ROS scavengers

Both the ROS scavenger Tempol and the peroxynitrite (ONOO<sup>-</sup>) scavenger Uric Acid (UA) induced a lower DHR-derived green fluorescence in *S. marinoi* cells exposed to the PUA tested (Fig. IV-3-6).. The highest scavenging effect in HEPTA-exposed *S. marinoi* cells compared to the PUA treated sample, was obtained with Tempol at 20 min ( $0.77 \pm 0.03$  fold decrease;  $p < 0.001$ ,  $n = 3$ ) (Fig.IV-3). The scavenging effect of Uric Acid at the same time point was  $0.58 \pm 0.03$  fold decrease ( $p < 0.001$ ,  $n = 3$ ; Fig.IV-3). A similar pattern was observed when samples were exposed to the MIX. Also in this case the scavenging effect of Tempol was stronger compared to that of Uric Acid. In particular, the highest decrease with respect to the PUA treated sample was always observed at 20 min, with Tempol inducing a  $1.77 \pm 0.09$  fold decrease ( $p < 0.001$ ,  $n = 3$ ), and Uric Acid a  $0.97 \pm 0.24$  fold decrease ( $p < 0.05$ ,  $n = 3$ ; Fig. IV-4). The same holds true for *S. marinoi* cells exposed to OCTA (Fig. IV- 5-6). Also in this case the highest decrease with respect to the control (i.e. no scavengers) was observed with Tempol at 20 min with the highest OCTA concentration (20  $\mu$ M), with  $1.11 \pm 0.24$  fold-decrease ( $p < 0.05$ ,  $n = 3$ ; Fig. IV-5), while the scavenging effect of Uric Acid was  $0.86 \pm 0.10$  fold decrease ( $p < 0.01$ ,  $n = 3$ ; Fig. IV-6). When OCTA-exposed *S. marinoi* cells were treated with the NO scavenger cPTIO, an opposite response was observed, with an increase in DHR-derived green fluorescence, both for OCTA 10  $\mu$ M and OCTA 20  $\mu$ M ( $p < 0.05$ ;  $n = 3$ )

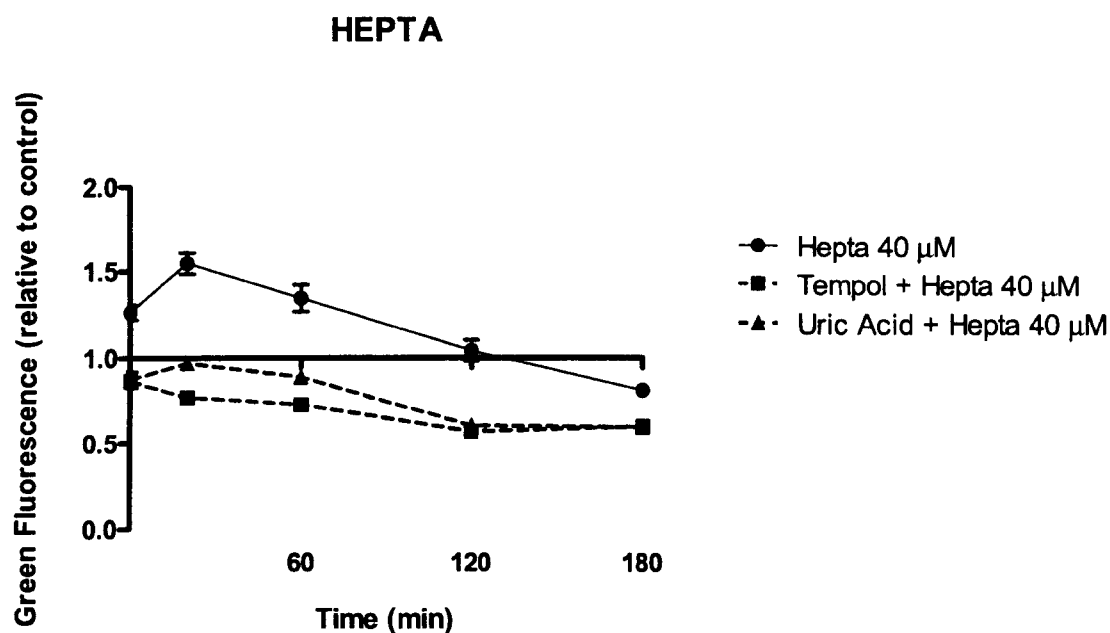


Figure IV- 3: *S. marinoi* cells exposed to HEPTA, the ROS scavenger Tempol and the peroxynitrite scavenger Uric Acid. Data are expressed in terms of DHR-derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates.

## MIX

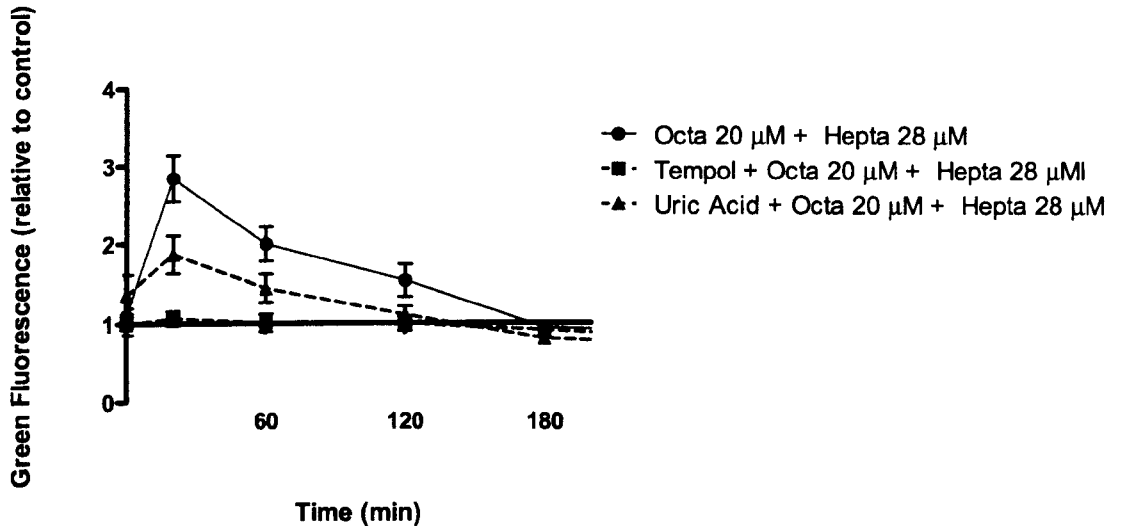


Figure IV- 4: *S. marinoi* cells exposed to OCTA, the ROS scavenger Tempol and the peroxynitrite scavenger Uric Acid. Data are expressed in terms of DHR-derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates.

## OCTA + Tempol

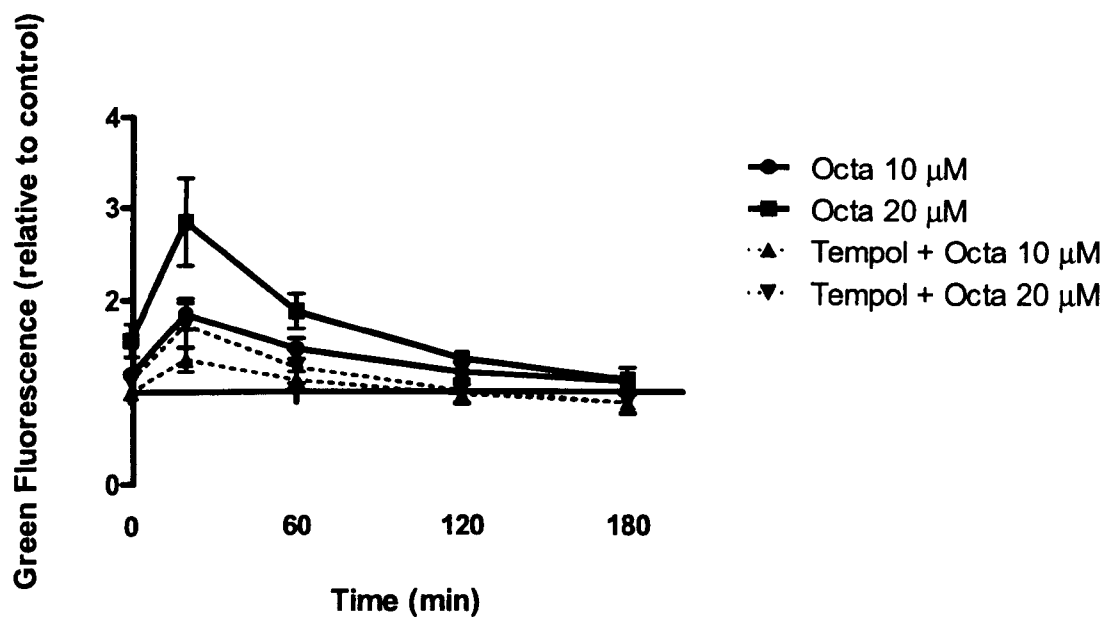


Figure IV- 5: *S. marinoi* cells exposed to different concentrations of OCTA and the ROS scavenger Tempol. Data are expressed in terms of DHR-derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates.

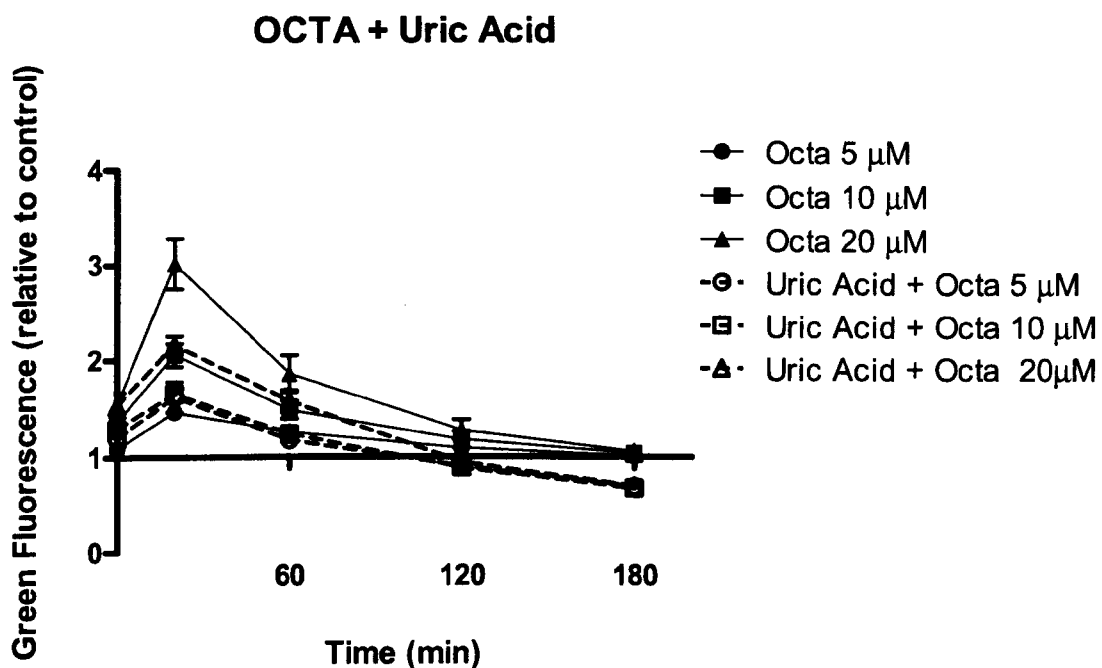


Figure IV- 6: *S. marinoi* cells exposed to different concentrations of OCTA and the peroxynitrite scavenger Uric Acid. Data are expressed in terms of DHR-derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates.

## OCTA + cPTIO

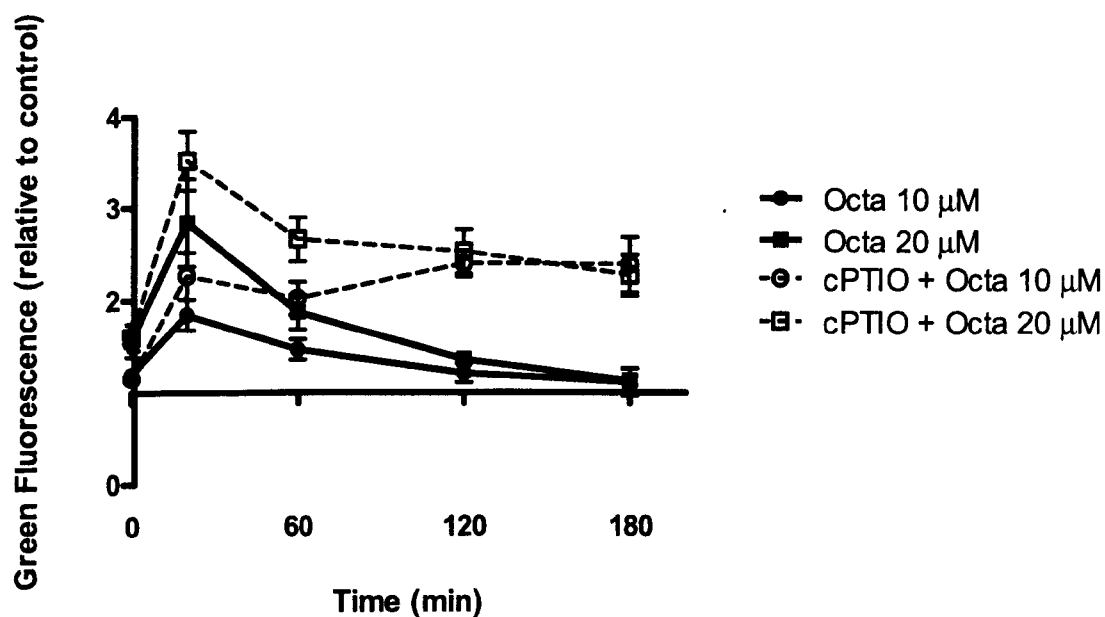


Figure IV- 7: *S. marinoi* cells exposed to different concentrations of OCTA and the NO scavenger cPTIO. Data are expressed in terms of DHR-derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates.

#### 4.3.2 NO inhibitors

In order to verify NO involvement and consequent peroxynitrite formation, OCTA-exposed *S. marinoi* cells were treated with different NO inhibitors. In particular LNAME, a NOS inhibitor, ST, a nitrate reductase (NR) inhibitor and DNAME, an inactive form of LNAME (i.e. a negative control for LNAME specificity), were used. Consistently to what was observed with the use of the NO scavenger, a higher DHR-derived green fluorescence was observed with all the NO inhibitors tested. Despite the fact that ST induced the lowest increase with respect to the PUA-treated sample (i.e.  $0.16 \pm 0.08$  fold increase, Fig. IV-8), this increase was the only one to be statistically significant ( $p < 0.01$ ,  $n = 3$ ). The fact that the increase induced by the NOS inhibitor LNAME was not significant might indicate a certain level of a-specificity of LNAME for NOS inhibition in *S. marinoi* cells. This is further supported by the fact that also in the presence of the inactive form DNAME an increase in DHR-derived green fluorescence was observed (Fig. IV-8). It is interesting to note that in Chapter 2 I have previously reported that enzymatic NO production in *S. marinoi* cells in the exponential phase of growth seems to be dependent on NR rather than NOS. This appears to be consistent with the fact that only the NR-inhibitor ST showed a statistically significant result.

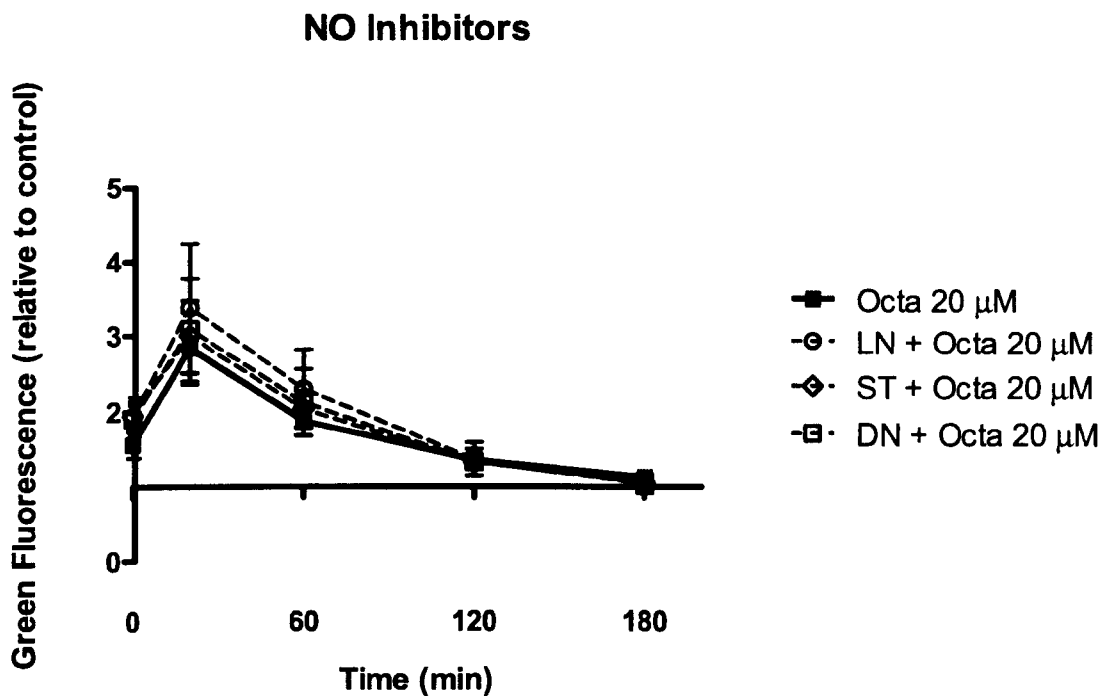


Figure IV- 8: *S.marinoi* cells exposed to OCTA and two different NO inhibitors: LNAME, a NOS inhibitor, ST, a NR inhibitor. DNAME is a negative control for LNAME, as it represents its inactive form. Data are expressed in terms of DHR-derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates.



#### 4.3.3 SOD inhibitors

In order to better elucidate the involvement of  $\text{H}_2\text{O}_2$ -mediated oxidation of DHR123, thus the formation of  $\text{H}_2\text{O}_2$  from  $\text{O}_2^-$  through the enzyme superoxide dismutase (SOD), two different SOD inhibitors were used, 2ME and DETC. In the presence of both inhibitors DHR-derived green fluorescence was significantly reduced (Fig. IV-9). The highest inhibition was obtained at 20 min with DETC, with a  $2.38 \pm 0.06$  fold decrease ( $p < 0.001$ ,  $n = 3$ ) compared to a  $1.43 \pm 0.56$  fold decrease obtained with 2ME ( $p < 0.05$ ,  $n = 3$ ), with respect to PUA treated samples (Fig. IV-9). These results indeed indicate SOD activity involvement and consequent  $\text{H}_2\text{O}_2$  formation (as well as  $\text{H}_2\text{O}_2$ -dependent downstream ROS generation) in the increase of DHR-derived green fluorescence in PUA-exposed *S. marinoi* cells.

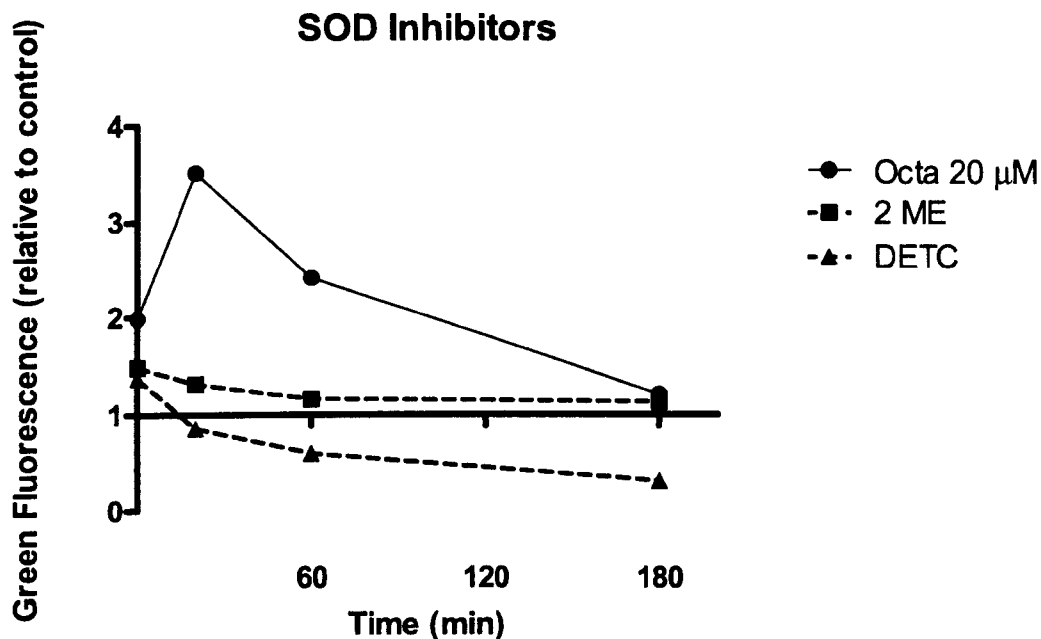


Figure IV- 9: *S. marinoi* cells exposed to OCTA and two different SOD inhibitors: 2ME and DETC. Data are expressed in terms of DHR-derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates.

#### 4.4 Discussion

From the use of different scavengers and inhibitors in assessing the oxidative response of *S. marinoi* to PUAs through the ROS/RNS sensitive dye DHR123, I have evidence of a potential involvement of different ROS and RNS.

It has been reported that the fluorescent dye DHR123 can respond to different types of oxidants, including  $H_2O_2$  in the presence of a metal or peroxidases (Royall and Ischiropoulos, 1993; McQuade and Lippard, 2010) and peroxynitrite ( $ONOO^-$ ) (Jourdeuil et al., 2001; Kooy et al., 1994). Therefore, at first it was chosen to apply two different types of scavengers, uric acid (UA), a  $ONOO^-$  scavenger (Gaupels et al., 2011), and Tempol, which has been reported to be a general reducing agent acting as a catalase-like agent (i.e. removing  $H_2O_2$  and preventing  $^{\bullet}OH$  formation) (Wilcox and Pearlman, 2008), even though it was also shown to attenuate  $ONOO^-$  (Khattab, 2006). The results obtained with all the different PUAs tested (i.e. OCTA, HEPTA and the MIX), indicate that both scavengers were having an effect in reducing DHR-derived green fluorescence in PUA-exposed *S. marinoi* cells (Fig. IV-3 to 6). This means that both  $ONOO^-$  and  $H_2O_2$  (and potentially the  $H_2O_2$ -derived hydroxyl radical) are involved in the oxidative stress response of *S. marinoi* to PUAs (see Chapter 3). It is interesting to note that with all the PUAs tested the effect of Tempol in reducing DHR-derived green fluorescence was always stronger compared to the effect of UA. Since Tempol is known to act mainly as a catalase-like agent (Wilcox and Pearlman, 2008), but also as a SOD-mimetic (i.e. metabolizing  $O_2^{\bullet -}$ ) (Patel et al., 2006) and as a  $ONOO^-$  scavenger (Carroll et al., 2000), the stronger effect is probably due to its multiple targets compared to the  $ONOO^-$  only scavenger UA (Ischiropoulos et al., 1999). At the same time, from these results it can also be inferred that the contribution of the ROS pathway is having a greater contribution with respect to the RNS pathway in the oxidative stress response of *S. marinoi* to PUAs.

It is interesting to note that the reaction between  $O_2^-$  and NO, leading to  $ONOO^-$  formation, has been linked to the toxicity of  $O_2^-$  or NO excess formation (Beckman et al., 1990; Radi et al., 1991). Another important aspect to be considered in terms of  $ONOO^-$  production *in vivo* is that both the timing and the site of  $O_2^-$  and NO production in cells might be different, and that they might overlap only for a limited time, so that  $ONOO^-$  formation will occur in excess of either NO or  $O_2^-$  (Grisham et al., 1999). In particular, NO is neutral and hydrophobic and is capable of crossing membranes, while  $O_2^-$  is anionic. As a consequence, the formation of  $ONOO^-$  will be likely to occur close to the sites of  $O_2^-$  formation (Alvarez and Radi, 2003; Gomes et al., 2006). A higher concentration of NO or  $O_2^-$  will create competition between  $ONOO^-$  formation and SOD activity for  $O_2^-$  (Halliwell and Gutteridge, 1999). The balance in the competition for  $O_2^-$  might be a key starting point for oxidative stress in different organisms (Lesser, 2006). For this reason, a pharmacological approach using both SOD inhibitors and inhibitors of NO biosynthesis was selected in order to better elucidate  $O_2^-$ , NO,  $ONOO^-$  and SOD involvement in the oxidative stress response of *S. marinoi* to PUAs.

Data obtained with two SOD inhibitors, 2ME and DETC, suggest an involvement of  $H_2O_2$ -dependent oxidation of DHR123, as previously reported (Ischiropoulos et al., 1999; Qin et al., 2008; Henderson and Chappell, 1993). SOD catalyzes the conversion of  $O_2^-$  to  $O_2$  and  $H_2O_2$  (Halliwell and Gutteridge, 1999). Both inhibitors induced a significant decrease of DHR-derived green fluorescence with respect to PUA treated samples (Fig. IV-9). This means that the formation of  $H_2O_2$  from  $O_2^-$  via SOD was prevented by the two inhibitors. It is interesting to note that Jourdeuil et al (2001) have previously reported that excess production of either NO or  $O_2^-$  inhibited the oxidation of DHR. However, these authors concluded that such response was due to an inhibition of peroxynitrite decomposition and relative DHR oxidation.

The use of the NO scavenger cPTIO, as well as of two different inhibitors for NO synthesis, i.e. a NOS inhibitor and a NR inhibitor, elicited an interesting response. In fact, in both cases, an increase of DHR-derived green fluorescence was observed (Fig. IV-7-8). It has been previously suggested that inhibition of nitric oxide synthesis in combination with DHR staining may lead to unexpected results (Ischiropoulos et al., 1999). In fact, by blocking or scavenging NO, the resulting unreacted superoxide can induce a (higher)  $H_2O_2$  formation and consequent dye oxidation via a peroxidase- or metal- dependent pathway (Ischiropoulos et al., 1999). However, if superoxide will be directed to other cellular targets and consequently will not form either  $ONOO^-$  or  $H_2O_2$ , then the DHR-derived fluorescence should not change (Ischiropoulos et al., 1999). The results reported here showed that NO inhibition and scavenging induced an increase in DHR-derived green fluorescence, therefore it is unlikely that the excess of superoxide was being directed away from  $H_2O_2$  and  $H_2O_2$ -derived species formation.

Both a NOS inhibitor and a NR inhibitor induced a similar response (i.e. increase in DHR-derived green fluorescence) in *S. marinoi*, suggesting that both enzymatic pathways were active in producing NO in *S. marinoi* cells exposed to OCTA (Fig. IV-8). I had previously observed that in *S. marinoi* cells under optimal growth conditions NO is produced mainly via NR and that NO is likely to function as a growth factor (see Chapter 2). It is however possible that in *S. marinoi* different NO enzymatic biosynthetic pathways are present depending on physiological vs. stress conditions (i.e. PUA exposure). It is interesting to note that it has been previously suggested that in algal cells NOS-dependent NO production could be present as an ‘active’ route, meaning that NO synthesis through this pathway should be highly regulated, for instance in the case of NO functioning as a messenger involved in signal transduction (Sakihama et al., 2002). However, it should also be noted that the use of the inactive form of the NOS inhibitor

LNAME, i.e. DNAME, also induced an increase in DHR-derived green fluorescence, even though not as strong as with LNAME. Being LNAME an inhibitor specific for the mammalian type of NOS, this could also indicate a certain level of a-specificity of LNAME for NOS inhibition in *S. marinoi* cells. It is also interesting to note that the increase of DHR-derived green fluorescence was higher in the presence of the NO scavenger cPTIO (Fig. IV-7) compared to NO inhibitors (Fig. IV-8). On the one hand this might be due to the fact that the incubation time for the inhibitors was not sufficient, whereas the NO scavenger was able to remove the majority of endogenous NO, thus increasing the availability of  $O_2^-$  directed towards  $H_2O_2$  production via SOD. On the other hand, if an involvement of both a NOS-like enzyme and the NR enzyme is hypothesized to drive NO biosynthesis in PUA-exposed *S. marinoi* cells, then it is possible that the use of one inhibitor at a time was not sufficient to block the synthesis of intracellular NO, and that the use of both inhibitors should have been combined.

Overall, it is possible to state that in our biological system DHR123, oxidation involves both a  $ONOO^-$  and a  $H_2O_2$ -driven process, with a major involvement of  $H_2O_2$ -related pathways, even if  $ONOO^-$  formation occurs as demonstrated by DHR oxidation inhibition through the  $ONOO^-$  scavenger UA (Table IV-1).

Table IV- 1: Schematic representation of the results on scavengers and inhibitors presented in Chapter 4. (↓) indicates a decrease in green fluorescence with respect to the PUA treated sample without the scavengers/inhibitors; (↑) indicates an increase in green fluorescence with respect to the PUA treated sample without the scavengers/inhibitors. Significance of the results is based on the Student's *t*-test; (\*)  $p < 0.05$ , (\*\*)  $p < 0.01$ ; (\*\*\*)  $p < 0.001$ , nt: not tested  $n = 3$ ,. ROS: reactive oxygen species; ONOO<sup>-</sup>: peroxynitrite; NO: nitric oxide; ST: sodium tungstate; SOD: superoxide dismutase.

	ROS scavenger	ONOO <sup>-</sup> scavenger	NO scavenger	NO inhibitor (ST)	SOD inhibitors
OCTA (20 μM)	↓ *	↓ **	↑ *	↑ **	↓ ****
HEPTA (40 μM)	↓ ****	↓ ****	nt	nt	nt
MIX (20 O + 28 μM H)	↓ ****	↓ *	nt	nt	nt

In Chapter 3 I observed a decrease in NO in *S. marinoi* cells in response to PUAs exposure. In light of the results reported here, it is possible to speculate that this decrease is due to a consumption of NO due to its reaction with excess  $O_2^-$ . Therefore, in terms of the specific ROS involved in the oxidative stress response of *S. marinoi* to PUAs, it is possible to hypothesize a major involvement of  $O_2^-$ ,  $H_2O_2$  as well as  $H_2O_2$ -derived species. In light of these considerations, an attempt to bring these findings into a biological context will now follow.

Biological transmission of a signal from an input stimulus to the appropriate physiological target is normally obtained through a signal transduction pathway, in which secondary messengers activate enzymes to propagate information (McQuade and Lippard, 2010). Secondary messengers are key components in the signalling cascade and can be of different nature, including oxidative compounds such as reactive oxygen (ROS) and reactive nitrogen (RNS) species (McQuade and Lippard, 2010). ROS as second messengers have been shown to be crucial for the expression of different transcription factors and signal transduction molecules such as heat-shock inducing factor, nuclear factor, the cell cycle gene *p53*, and mitogen-activated protein kinase (Martindale and Holbrook, 2002; Lesser, 2006). It is interesting to note that bacteria and yeast induce distinct defence proteins in response to either  $O_2^-$  or  $H_2O_2$  production, even though a substantial overlap exists between the two responses (Demple, 1991; Jamieson, 1992). It is possible, however, that  $O_2^-$  and  $H_2O_2$  can act independently as signalling molecules (Vranova et al., 2002). In plants, ROS signalling has been linked to plant defences responses, growth and morphogenesis as well as cell death, through both programmed cell death and necrosis (Vranova et al., 2002). Overall, ROS can act as ubiquitous signal molecules and the intracellular (but also extracellular) level of ROS will determine the



different type of responses. At low concentrations ROS can induce defence genes and adaptive responses (Chamnongpol et al., 1998; Levine et al., 1994; Green and Fluhr, 1995), whereas at high concentrations cell death is initiated (Levine et al., 1994; Desikan et al., 1998; Vranova et al., 2002). A similar type of mechanism might also be expected to be present in *S. marinoi* response to PUAs. Indeed, it has been previously hypothesized that the RNS NO can act as both an intracellular and intercellular signalling molecule in the stress response of the marine diatom *Phaeodactylum tricornutum* to PUA exposure (Vardi et al., 2006); thus a similar mode of action is likely to be present also in *S. marinoi* with ROS.

My results confirm that specific ROS are produced in *S. marinoi* upon exposure to PUAs. In *S. marinoi* ROS (e.g.  $O_2^-$ ,  $H_2O_2$ ) might have a multifunctional effect inducing the enhancement of PUAs allelopathic effects directed towards different phytoplankton species, which would require ROS release, as well as have a regulatory role as signalling molecules within *S. marinoi* populations. Indeed, Fontana et al. (2007b) found that a highly reactive oxygen species burst (hROS, e.g.  $ONOO^-$  and  $^{\bullet}OH$ ) follows the activation of the lipoxygenase (LOX)-mediated oxidation of polyunsaturated fatty acids (PUFA), leading to fatty acid hydroperoxides (FAHs) synthesis. The authors speculate that both classes of compounds, hROS and FAHs, act together in the pro-apoptotic and teratogenic effects directed towards copepods, i.e. the main diatom grazers. Moreover, it has been long documented that raphidophycean flagellates produce ROS, including  $O_2^-$ ,  $H_2O_2$  and  $^{\bullet}OH$  (Tanaka et al., 1992; Oda et al., 1992a; Oda et al., 1992b; Kawano et al., 1996), and that these reactive species are linked to their ichthyotoxicity (Tanaka et al., 1992; Yang et al., 1995; Marshall et al., 2003). It has been suggested that ROS can act in combination with other factors, such as free fatty acids, in causing the reported ichthyotoxic effects (Okaichi et al., 1989; Marshall et al., 2003; Marshall et al., 2005). In particular, in *C.*

*marina* it has then been suggested that production and release of ROS could function as a defence mechanism involved in allelopathic interactions in combinations with PUFAs, for instance to prevent bacterial fouling or in the competition with other microalgae (Marshall et al., 2005). It can also be speculated that in PUA-exposed *S. marinoi* cells at low PUA concentrations the intracellular antioxidant system is able to cope with the moderate excess of ROS production (e.g.  $O_2^-$  and  $H_2O_2$ ), whereas at higher concentrations it cannot, thus resulting in the detection of an excess production of both  $O_2^-$  and  $H_2O_2$ . Indeed, DHR-derived green fluorescence was proportional to PUA concentrations (Chapter 3). In the freshwater green alga *Chlamydomonas moewusii* paraquat exposure was found to induce an increase in production of both  $O_2^-$  and  $H_2O_2$  in non-chlorotic cells (Prado et al., 2012). Interestingly, in this study, the lowest paraquat concentration induced only  $O_2^-$  increase with  $H_2O_2$  levels (assessed with DHR123) similar to the control, whereas at the higher concentrations both ROS were found to increase. Production of excess  $O_2^-$  results in the generation of  $H_2O_2$  by spontaneous or enzymatic dismutation through SOD (Perl-Treves and Perl, 2002). In the case of *C. moewusii* the authors suggested that at low paraquat concentrations the intracellular antioxidant systems may not have been able to detoxify excess  $O_2^-$  but pathways for the removal of the relative amount of  $H_2O_2$  formed could have been sufficient (Prado et al., 2012). On the contrary, the antioxidant cellular capacity might have been overwhelmed after the exposure to high herbicide concentrations, thus resulting in detection of excess production of both  $O_2^-$  and  $H_2O_2$  (Prado et al., 2012). Given that based on the results reported in this study, a major involvement of  $O_2^-$  and  $H_2O_2$ -driven oxidative responses have been found in PUA-exposed *S. marinoi*, it could be hypothesized that in a PUA-producing species, such as *S. marinoi*, which would be naturally more exposed to PUAs at sea, specific antioxidant enzymatic systems (i.e. SOD and CAT) might play a key role in the oxidative stress

response to PUAs in comparison to a non-PUA producing species. In N2A cells pre-treated with Vitamin E and SOD/CAT, the cyanotoxin saxitoxin induced a lower oxidative stress with respect to cells with no antioxidant treatment (Melegari et al., 2012). Additionally, the insecticide cypermethrin against copepod parasites and the antifouling compound triphenyltin were able to induce an increase in SOD, CAT and GPX activities in different microalgal species (Wang Z.H. et al., 2011; Xu et al., 2011), providing evidence that these antioxidant enzymes are involved in the oxidative stress response to different chemical factors. In terms of intracellular scavenging mechanisms active in marine phytoplankton, a recent paper by Portune et al. (2010) was one of the few studies to try to relate and compare ROS production with the activity of the two well-known antioxidant enzymes SOD and CAT. Their findings reported a correlation between  $O_2^-$  per cell and SOD activity per cell during the growth phase only in three of the six raphidopythes tested, possibly due to different cellular locations of  $O_2^-$  radicals and SOD enzymes (Portune et al., 2010). Increased CAT activity in correlation with  $H_2O_2$  per cell was also observed in three out of six species analysed. In particular, it was observed that SOD as well as CAT appear to play a major role during the early exponential growth phase, while during later growth phases other antioxidants such as APX and GPX may have a greater role in scavenging ROS (Portune et al., 2010). In the marine diatom *Thalassiosira pseudonana* it was found that a short excess light treatment in high-light-adapted cells was able to increase SOD and APX activities and to induce the expression of some key APX and SOD genes, with respect to low-light grown cells (Waring et al., 2010). This would imply that growth at high light conditions predisposes cells to enhance their scavenging capacity for  $O_2^-$  and  $H_2O_2$  (Waring et al., 2010).

Given that an excess production of  $O_2^-$  is linked to the generation of downstream ROS and ROS-mediated effects, and that based on the results reported here from *S.*

*marinoi*, a major involvement of  $O_2^-$ ,  $H_2O_2$  as well as  $H_2O_2$ -derived species has been hypothesized, a key point is represented by the possible cellular mechanisms of  $O_2^-$  and  $H_2O_2$  generation.  $O_2^-$  is a metabolic byproduct of aerobic respiration and oxygenic photosynthesis (Fridovich, 1998; Falkowski and Raven, 1997). Additionally, in raphidophycean flagellates,  $O_2^-$  production has been linked to an enzymatic system located on the cell surface, and that such a system is closely related to the human neutrophil NAD(P)H oxidase (NOXs) which is a well known enzymatic system of  $O_2^-$  generation (Kim et al., 2000). NOXs are a family of enzymes consisting of flavoprotein, cytochrome *b* and regulatory subunits specific to the family member (Lambeth, 2004). They can be distributed in different tissues and when they are activated they assemble in the cell membrane and reduce oxygen to superoxide (Lambeth, 2004). Marshall et al. (2002) have suggested that the reducing power to generate the NADPH-dependent  $O_2^-$  could be supported through the photosynthetic electron transfer, which would sustain  $O_2^-$  production at the cell membrane. NOXs are mainly studied in phagocytic cells in relation to the defence mechanism against invading pathogens (Nauseef, 2007; Cross and Segal, 2004), and they are known to be activated by different stimuli, including fatty acids (Kakinuma and Minakami, 1978), lectins (Cohen et al., 1980), and immunoglobulins (Kiyotaki et al., 1978).

Understanding the localization of sites of both RNS and ROS production is also an important aspect to be considered in order to better understand the effects that such reactive species will have at the cellular level. Vardi et al. (2006) have indicated that in *P. tricornutum* DAF-FM DA derived fluorescence did not localize neither in the chloroplasts nor in the nucleus, although it appeared to be closely associated with the latter. In terms of DHR123 localization, it is known that it localises at the level of mitochondria (Prado et al., 2012). Interestingly, a recent paper investigated  $H_2O_2$

localization in the green alga *Microcystis* after salt and osmotic stress by means of the dye H<sub>2</sub>DCFDA and confocal laser scanning microscopy (Darehshouri and Lütz-Meindl, 2010). These authors reported that the majority of H<sub>2</sub>O<sub>2</sub> production occurred at the level of chloroplasts, mitochondria and the cytoplasm. Although the aim of this experimental section has not been to relate ROS production and cellular localization of dyes, epifluorescence pictures of dye-stained cells are provided in this thesis in order to visualize the presence of the different fluorescent dyes used within the cells (see Chapter 3 and Chapter 5). For a better understanding of dye staining and localisation, it would be useful to combine epifluorescence microscopy with confocal microscopy in order to localize RNS and ROS more precisely inside the cells

As a conclusive, general remark, what it is considered to be a major topic in the field of ROS and RNS research in biological systems is how to best assess the significance of specific ROS and RNS, in order to understand the nature and effect of each particular ROS or RNS in a biological context (Murphy et al.; 2011; Winterbourn, 2008). The biological impact of ROS is thought to be highly dependent on the specific molecules involved and on the microenvironment and physiological context in which they are being produced (Murphy et al., 2011). It is therefore important that in these types of studies an effort is made to indicate the potential particular ROS thought to be involved and the reactions responsible for the hypothesized biological effects (Murphy et al., 2011).

## CHAPTER 5

### Expression of Death-Specific Protein Genes (*DSPs*) in Response to PUAs in *Skeletonema tropicum*

#### 5.1 Introduction

*Skeletonema costatum* (sensu lato) is one of the most widespread genera of bloom-forming coastal marine diatoms, and it has been reported to be composed of several morphologically and genetically diverse species (Sarno et al., 2007; Sarno et al., 2005; Zingone et al., 2005). Genetic analysis based on partial sequences from nuclear small subunit ribosomal DNA (LSU rDNA), allowed for the identification of new *Skeletonema* species as well as for the study of their biogeography (Kooistra et al., 2008; Sarno et al., 2007; Sarno et al., 2005). *Skeletonema tropicum* CLEVE was first described and identified in the tropical waters of the Atlantic Ocean and Caribbean coast of South America (Cleve, 1900). Subsequent reports of *S. tropicum* include the coast of eastern USA, the Atlantic side of Panama, Guyana and Surinam, the East and South China Seas (Cheng et al., 2008; Cheng and Kiu, 1992), coastal Japan (Ueno, 1993), Gulf of Mexico (Castillo et al., 1995), Italy (Sarno et al., 2005), the western side of Panama (Kooistra et al., 2008), southern Brazil (Bergesch et al., 2009), and India (Naik et al., 2010). In China, *S. tropicum* has been observed in the tropical and subtropical waters of the East and South China Seas, including Wenzhou, Xiamen, Daya Bay, Taiwan Straits and Haikou (Cheng et al., 2008; Cheng and Kiu, 1992; Kooistra et al., 2008), as well as the temperate waters of Jaozhou Bay in the Yellow Sea (Liu et al., 2012). The so-called *S. costatum* Kao strain was isolated from Kaoshiung, between the Taiwan Strait and the South China

Sea. This strain has now been reassigned as *tropicum* based on LSU rDNA sequence analysis (Sarno and Minucci, pers. comm.) and on 18S rRNA analysis (Mr. Hung, pers. comm.). In this strain, two novel genes encoding two death-specific proteins (*ScDSP-1* and *ScDSP-2*) thought to be involved in the stress-dependent cell death machinery have been characterized (Chung et al., 2008; Chung et al., 2005).

Self-induced cell death is best known and studied in relation to programmed cell death (PCD) in metazoans (Leist and Nicotera, 1997). PCD is an active and genetically regulated type of cell death, and in multicellular organisms it contributes to several processes, including morphogenesis, the removal of damaged or mutated cells, the development of the immune and nervous system, the protection against infections and in maintaining organism functionality (Danial and Korsmeyer, 2004). In the context of metazoans, the role and importance of PCD is well understood as a form of cooperation, or 'altruistic' behaviour, that negatively affects the lower level (the cell), but allows the survival of the higher level, (the organ) (Williams, 1991). Different PCD-like processes are recognized in multicellular organisms, including for instance apoptosis, autophagy and paraptosis (Kroemer et al., 2009; Sperandio et al., 2000). Apoptosis is a specific PCD morphotype characterized by distinct morphological and biochemical features such as chromatin condensation, nuclear fragmentation, mitochondrial depolarization, cell shrinkage, plasma membrane blebbing and the activation of a family of cysteine proteases termed caspases (cysteine-dependent aspartate-directed proteases), which are the main regulators of the cell death process (Kerr et al., 1972). Paraptosis is a different PCD type, that was discovered relatively recently in cells during neurodegeneration, and which is characterized by cytoplasmic vacuolization, mitochondrial swelling, but without any other morphological hallmark of apoptosis, and it has been reported to be mediated by mitogen-activated protein kinases (MAPKs) (Sperandio et al., 2000; Sperandio et al.,

2004). Autophagy's main characteristics are instead an extensive cytoplasm vacuolization, accumulation of autophagic vacuoles and the absence of chromatin condensation (Kroemer et al., 2009). In contrast to these forms of PCD, necrosis is defined as an injury-dependent form of cell death characterized by organelle and cytoplasmic swelling, rupture of the plasma membrane with the external release of the cellular contents and without any of the biomolecular hallmarks of apoptosis (Kroemer et al., 2009).

Apoptosis has been mainly studied in metazoans, but both vascular plants (e.g. Greenberg, 1996; Pennell and Lamb, 1997; Lam et al., 2001) and some unicellular eukaryotic and prokaryotic organisms have been found to display apoptotic-like features. An early observation that a PCD-like mechanism could be present in phytoplankton, was made in cells of the freshwater diatom *Asterionella formosa* infected by a parasite, which rapidly died before spreading the parasite to neighbouring cells (Canter and Jaworski, 1979). Since then, unicellular organisms reported to display an apoptotic-like cell death, include chlorophytes (Berges and Falkowski, 1998; Segovia et al., 2003; Segovia and Berges, 2005), coccolithophores (e.g. Bidle et al., 2007), dinoflagellates (Vardi et al., 1999; Franklin and Berges, 2004; Segovia, 2007; Dunn et al., 2004), diatoms (Casotti et al., 2005; Brussaard et al., 1997), yeast (Frohlich and Madeo, 2000), kinetoplastids and slime moulds (Cornillon et al., 1994), and bacteria (Lewis, 2000), including cyanobacteria (Berman-Frank et al., 2004).

The concept of active, self-induced cell death in the context of unicellular organisms is still considered a matter of ongoing debate (Deponete, 2008; Jimenez et al., 2009). The key point is that in unicellular individuals cell death results in the complete loss of the organism. Therefore, the theory of an evolved altruistic behaviour that can benefit the whole organism while being detrimental for a part of it, might appear contradictory and it is not obvious to what extent such concept can be invoked to explain PCD in unicellular



organisms (Nedelcu et al., 2010). In the perspective of the evolutionary drivers responsible for the maintenance of PCD in phytoplankton (and unicellular organisms, in general), different hypotheses have been proposed (Bidle and Falkowski, 2004). These include the idea that PCD can be an altruistic adaptation evolved in order to benefit a population by removing damaged cells and supplying surviving ones with organic compounds, as it has been proposed for bacteria and yeast (Lewis, 2000; Frohlich and Madeo, 2000). In the case of phytoplankton, PCD might be a way to eliminate ageing cells and to cope with nutrient stress at the population level when conditions become unfavourable, as, for instance, in the final stages of blooms (Bidle and Falkowski, 2004; Vardi et al., 1999; Vardi et al., 2007; Vardi et al., 2006). Additionally, it has been suggested that the presence of PCD in unicellular individuals might provide a benefit through an increased genetic fitness (Bidle and Falkowski, 2004; Franklin et al., 2006). In particular, it is hypothesized that a particular process can become more frequent and established if it has a positive effect on the fitness of genes, either present in an individual or in a closely related kin (i.e. the kin selection model) (Hamilton, 1964a; Hamilton, 1964b). PCD-related genes might also have been maintained at low level of expression in a co-evolving manner with separate metabolic pathways, becoming overexpressed only under specific stressful conditions (Bidle and Falkowski, 2004). An alternative explanation for PCD evolution in unicellular organisms is instead related to the role of viruses (e.g. Bidle et al., 2007). On one hand it is believed that PCD can be a mechanism through which viral multiplication and diffusion is limited during infection. Indeed, viral proteins have been shown to have different regulatory functions in animal apoptosis (Teodoro and Branton, 1997), and also to be able to induce caspases (e.g. Liu et al., 2001). On the other hand, viral infection itself might have passed death-related genes via later gene transfer to the hosts (Bidle and Falkowski, 2004). It is known that persistent viral

infections (lysogenic cycle) have a key function in determining host evolution (Villarreal, 2001). Therefore, viruses in a lysogenic state can be inactive and persist indefinitely until an external signal triggers the conversion to the lytic pathways (Bidle and Falkowski, 2004). An alternative scenario, not linked to an altruistic type of PCD evolution in unicellular organisms, has instead been proposed by Nedelcu et al. (2010). These authors propose that PCD might not be an altruistic adaptation but rather that it might have been retained as a maladaptive trait in the form of a byproduct of selection related to pro-survival functions, and that it could have evolved into an altruistic trait in the presence of specific conditions leading to group selection (Nedelcu et al., 2010). Another interesting hypothesis reported for yeast is related to unsuccessful mating and pheromone signalling (Buttner et al., 2006). In yeast, pheromone signalling has been shown to lead to apoptotic death in cells that are not capable to mate. In this view, PCD might function as a way to remove haploid cells while increasing the survival of diploid cells, ultimately enhancing genetic diversity through meiotic recombination (Buttner et al., 2006).

The central component of the apoptotic machinery is represented by a family of proteases known as caspases. The enzymatic activity of caspases is initiated in response to proapoptotic signals and leads to an enzymatic cascade resulting in the cleavage of a set of proteins with the ultimate disassembly of the cell (Thornberry and Lazebnik, 1998). Caspases have only been identified in multicellular organisms (Cohen, 1997), but caspase activity has also been shown in vascular plants during hypersensitive response (del Pozo and Lam, 1998), in yeast (Madeo et al., 2002) and in trypanosomes (Szallies et al., 2002). Two different families of caspase orthologues, paracaspases and metacaspases, have been identified in morphologically diverse organisms. In particular, paracaspases were found in animals and slime moulds, while metacaspases were identified in plants, fungi, unicellular protozoa and different bacterial species (Uren et al., 2000). The main

characteristic of caspases is their high specificity and very selective requirement for cleavage after an aspartic acid residue and the recognition of at least four amino acid terminals to the cleavage site (Thornberry, 1998). Such high specificity of caspases ensures that during PCD there is no uncontrolled protein digestion, but that only a specific set of proteins will be cleaved in a regulated manner, usually at a single site (Fischer et al., 2003). Metacaspase orthologues and proteases with caspase-like activity have been identified in both prokaryotic and eukaryotic phytoplankton, including cyanobacteria (e.g. Berman-Frank et al., 2004), the chlorophyte *Chlamydomonas reinhardtii* (Shrager et al., 2003; Merchant et al., 2007) and *Dunaliella tertiolecta* (Segovia and Berges, 2009), the marine diatom *Thalassiosira pseudonana* (Bidle and Bender, 2008; Thamatrakoln et al., 2012) and *Phaeodactylum tricornutum* (Vardi et al., 2008) and the marine haptophyte *Emiliana huxley* (Bidle et al., 2007). From a phylogenetic point of view, phytoplankton metacaspases from both the 'red' (*T. pseudonana*, *E. Huxley* and *P. tricornutum*) and 'green' (*C. reinhardtii* and *D. tertiolecta*) lineages have been found to group with metacaspases of fungi, trypanosomes and higher plants, possibly indicating that they share similar roles (Bidle and Falkowski, 2004); on the other hand, cyanobacterial metacaspase orthologues are more differentiated, which might be linked to an acquisition by lateral gene transfer (Bidle and Falkowski, 2004).

The specific mode of action in which PCD is initiated in unicellular organisms remains to be elucidated. Nitric Oxide (NO) has been shown to be involved in the induction of PCD and PCD-like processes in different biological systems (Besson-Bard et al., 2008; Arasimowicz-Jelonek et al., 2012; Vardi et al., 2008; Ali et al., 2010). Additionally, reactive oxygen species (ROS) have been shown to have an important role in inducing PCD in a wide range of organisms from bacteria, to mammalian and plant cells (for reviews see Andrianasolo et al., 2007; Scherz-Shouval and Elazar, 2007; Jones,

2008; Kuwabara et al., 2008; Lam, 2008; Rockenfeller and Madeo, 2008). Cellular responses to ROS are strongly dependent on ROS concentration, and may range from up-regulation of antioxidants to cell death by necrosis (Chandra et al., 2000). Between these two extremes, intermediate ROS concentrations have been reported to induce PCD or apoptosis (Korsmeyer et al., 1995; Levine et al., 1996). Additional factors influencing the wide spectrum of cellular responses to ROS include the severity of the damage, the cell type, the magnitude of the stress stimulus and the duration of the exposure (Martindale and Holbrook, 2002). Two possible mechanisms have been reported to explain the RNS/ROS-mediated switch between apoptosis and necrosis (Chandra et al., 2000). The first one involves the inactivation of caspases due to the oxidation of their active site thiol group by oxidants (Samali et al., 1999), or S-nitrosylation can be responsible for inducing a necrosis-like cell death (Melino et al., 1997). Alternatively, another mechanism could be explained by a decrease in cellular levels of ATP due to failure of mitochondrial energy production in a condition of oxidative stress (Leist et al., 1999; Tsujimoto et al., 1997). Different case studies have related the occurrence of ROS and PCD in marine phytoplankton (e.g. Butow et al., 1997; Vardi et al., 1999; Segovia and Berges, 2009). In the dinoflagellate *Peridinium gatunense* PCD leading to the collapse of the algal population was found to be mediated by oxidative stress induced by CO<sub>2</sub> limitation (Vardi et al., 1999). A similar finding of ROS-mediated cell death was found in a *P. gatunense* population at the end of a bloom, despite an increase in antioxidant activity (Butow et al., 1997). Additionally, apoptotic-like cell death concomitant with accumulation of ROS due to the toxic cyanobacterium *Microcystis* sp. (Vardi et al., 2002), as well as application of H<sub>2</sub>O<sub>2</sub> (Vardi et al., 2007) has been reported. In the chlorophyte *Dunaliella tertiolecta* dark-induced apoptosis involving the activity of caspase-like enzymes has been linked to an increase in ROS production (Segovia and Berges, 2009).

Finally, in the prymnesiophyte *Emiliana huxley* viral infection has been associated to an enhanced production of ROS, possibly leading to PCD (Evans et al., 2006).

In terms of potential triggers reported to induce PCD-like processes in marine phytoplankton, one of the first observations made in the marine diatom *Thalassiosira weissflogii* and in the chlorophyte *Dunaliella tertiolecta* reported that both nitrogen and light limitation could induce the activity of proteases (Berges and Falkowski, 1998). Iron starvation has also been shown to activate metacaspases and PCD in the marine diatom *Thalassiosira pseudonana* (Bidle and Bender, 2008; Thametrakoln et al., 2012), while in the marine cyanobacterium *Trichodesmium* spp. the PCD pathway was induced by both iron and phosphorus starvation, as well as high irradiance and oxidative stress (Berman-Frank et al., 2004). In the chlorophyte *Dunaliella tertiolecta* caspase-like activity was associated to a darkness condition (Segovia et al., 2003), and in a subsequent study the inhibition of such enzymatic activity was found to prevent ROS-mediated dark-induced apoptosis (Segovia and Berges, 2009). Marine viruses have also been reported to be one of the major triggers of phytoplankton mortality at sea (Fuhrman, 1999; Bidle and Vardi, 2011). In particular, it has been suggested that an interaction exists between autocatalytic cell death and lytic viral infection in the coccolithophorid *Emiliana huxley*, with an upregulation of metacaspases expression and induction of caspase-like activity (Bidle et al., 2007). Later, it has been found that viral glycosphingolipids are responsible for the lytic infection and PCD in *E. huxley* (Vardi et al., 2009). The collapse of the annual bloom of the dinoflagellate *Peridinium gatunense* was found to be induced by CO<sub>2</sub> limitation followed by an increase in ROS production (Vardi et al., 1999). In the same study, an inhibitor of proteases was found to stimulate cyst formation preventing cells to die (Vardi et al., 1999). The authors speculated that the evolution of PCD might be possibly linked to a mechanism by which only the fittest individual are able to encyst,

while unhealthy individuals would be eliminated, thus conferring a selective advantage to the community over the course of time (Vardi et al., 1999). In another study, a protease excreted by *P. gatunense* senescing cells was found to sensitize cells in the logarithmic phase of growth to oxidative stress and to lead to premature massive cell death (Vardi et al., 2007). In the raphidophyte *Heterosigma akashiwo* different levels of heat stress were able to induce a range of responses, going from recovery, to PCD-induction and finally to necrotic death when the stress was too severe (Dingman and Lawrence, 2012). In the prokaryotic cyanobacterium *Anabena* sp., salt stress has also been found to induce PCD (Ning et al., 2002), while in the marine cyanobacterium, *Trichodesmium* sp., increased production of extracellular polysaccharide aggregates, defined as transparent exopolymeric particles (TEP), was found to induce PCD (Berman-Frank et al., 2007). In this study, PCD induction was related to high caspase-like activity and occurred under iron and phosphorus starvation, high irradiance and oxidative stress (Berman-Frank et al., 2007). With respect to the possible effect of secondary metabolites in inducing autocatalytic cell death in marine phytoplankton, an early observation in the cultures of the dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge, showed contact inhibition at low cell densities, suggesting that there could be a mechanism of autocontrol in preventing cell proliferation in the natural environment (Costas et al., 1993). In the marine diatom *Thalassiosira weissflogii*, diatom-derived polyunsaturated aldehydes (PUAs) were shown to induce apoptotic-like cell death, suggesting for a possible role of PUAs in activating the cell death cascade and in determining changes of population dynamics and the ecology of species succession (Casotti et al., 2005). Additionally, in the marine diatom *Phaeodactylum tricornutum*, PUA exposure resulted in an altered expression of metacaspases through the overexpression of a gene associated to NO generation (Vardi et al., 2008). Interestingly, the secondary metabolite Euplotin C derived

from the marine ciliated protist *Euplotes crassus*, was found to induce PCD in the congeneric *Euplotes vannus*, which does not produce this metabolite (Cervia et al., 2009). These findings suggest that specific secondary metabolites can play an ecological role in broadening phytoplankton niche size through different mechanisms, including for instance autocatalytic induced cell death (Cervia et al., 2009). Additional phytoplankton-derived compounds that were found to induce apoptosis in insect and human cell lines, include extracellular organic compounds from the ichthyotoxic red tide alga *Heterosigma akashiwo* (Twiner et al., 2005) and domoic acid (Pinto-Silva et al., 2008).

In the marine diatom *S. tropicum*, it has been identified a gene coding for a calcium-regulated protein, named *Skeletonema costatum* death specific protein (*ScDSP*) (Chung et al., 2005), which is characterized by a transmembrane domain and a pair of EF-hand motifs, typical of calcium regulated proteins (Luan et al., 2002; Means and Dedman, 1980). *ScDSP* gene expression was shown to be strongly upregulated in dying cells and during light stress, suggesting for a possible role of the encoded protein in the signal transduction of stress to the cell death machinery (Chung et al., 2008; Chung et al., 2005). In particular, *ScDSP* expression has been related to the blockage of the electron flow between PSII and cytochrome b6f, by the use of different photoinhibitors and light manipulation treatments, and it was found to be nitric oxide (NO) dependent (Chung et al., 2008). Based on these findings, the aim of the following experimental section has been to test, in *S. tropicum* (Kao strain), the effect of the diatom-derived secondary metabolites PUAs on the expression of the two death-related genes *ScDSP-1* and *ScDSP-2* identified by Chung et al. (2005, 2008), and the possible involvement of ROS production associated with *ScDSP* expression.

## 5.2 Materials and Methods

### 5.2.1 Culture Conditions

A unialgal culture of *Skeletonema costatum* (now reassigned *tropicum*, Sarno and Minucci, pers. comm.) strain Kao (from Kaoshiung, Taiwan) was grown at 23°C on a 12h-12h light-dark cycle under a photon flux density of 110  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . Natural seawater, amended with f/2 nutrients (Guillard, 1975) was used as medium.

### 5.2.2 Preparation of Chemicals

For polyunsaturated aldehydes (PUAs) preparation see Materials and Methods 3.1.2.2, Chapter 3.

As a ROS donor, 30 wt. % hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (Sigma Aldrich, Milan, Italy) was used. A predilution of 1/200 of  $\text{H}_2\text{O}_2$  was prepared and it was added to samples at the ratio of 50  $\mu\text{L}$ :1 mL.

For ROS detection *in vivo* the fluorescent ROS-sensitive dye DHR123 was used (see Materials and Methods 3.2.2.2, Chapter 3).

The ROS scavenger 4-Hydroxy-TEMPO (Tempol) (Sigma-Aldrich, Milan, Italy) was used at final working concentrations of 5 mM, dissolved in filtered sea water (FSW). The superoxide dismutase (SOD) inhibitor sodium diethyldithiocarbamate trihydrate (DETC) (Sigma-Aldrich, Milan, Italy) was also used at final working concentration of 1 mM DETC dissolved in FSW. Samples were incubated for 120 min before PUAs addition.



### 5.2.3 Statistical Analysis

Statistical significance with respect to control values was evaluated with the Student's *t*-test using the Excel spreadsheet (Microsoft Office).

### 5.2.3 PUA Toxicity Test

The EC50 concentrations for the different PUAs (i.e. OCTA and HEPTA) were empirically determined from the relationship between growth rate and the PUAs concentration (Fig. V-1). Growth rates were calculated as  $\mu$  (day<sup>-1</sup>) according to

$$\mu = \ln [(N_1/N_0)/t] \quad (1)$$

where  $N_0$  and  $N_1$  represent cell density at the start and the end of the growth period, and  $t$  is the time between measurements (in days).

Cell concentrations were determined by placing 1 mL of algal culture into a Sedgwick-Rafter counting chamber (Hausser Scientific Partnership) and cells were examined with a light microscope (BX60; Olympus). Microscopy counts were obtained from at least 200 cells at 20 x magnification. The EC50 for growth at 24 h was therefore considered to be 8  $\mu$ M for OCTA, and 18  $\mu$ M for HEPTA. The following gene expression experiments were performed using three PUA concentrations representing the EC50, half the EC50 and twice the EC50 concentration for OCTA and the EC50 and twice the EC50 for HEPTA. Two different sets of experiments were performed: one for gene expression and the other for ROS detection.

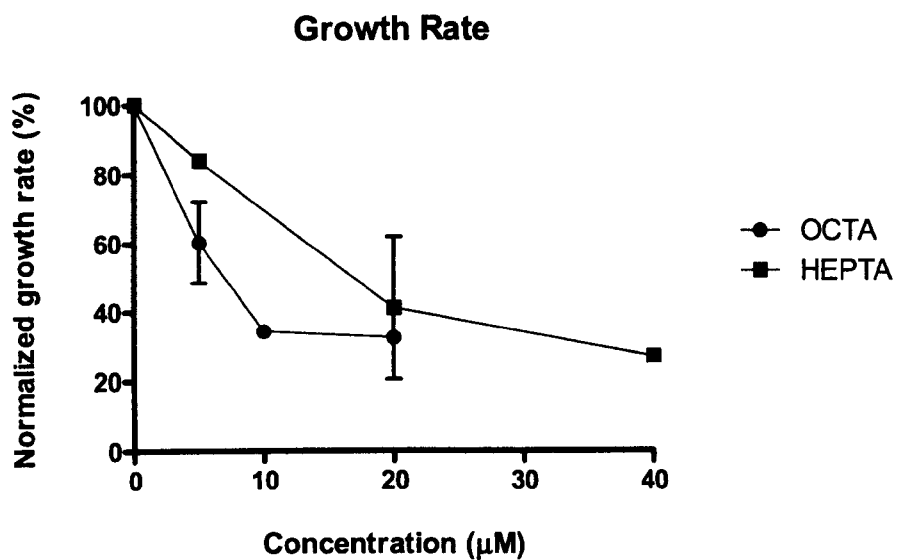


Figure V- 1: Growth rate (normalized to the control) vs. PUA concentrations (μM). The plot has been used to empirically estimate the EC50 concentrations for growth at 24 h.

#### 5.2.4 Experimental Setup

For gene expression measurements, *S. tropicum* was grown in 10 L polycarbonate carboys (Nalgene). When the culture reached different growth stages (either the early or the late exponential), cells were transferred into 2 L polycarbonate bottles (Nalgene), and divided into PUA-treated samples, an H<sub>2</sub>O<sub>2</sub> treated sample and the control, which were sampled at different time points for total RNA extraction and cell counts. Specific combinations of PUAs concentrations and time intervals were selected for each experiment. Namely, chosen concentrations were half the EC<sub>50</sub>, the EC<sub>50</sub> and double the EC<sub>50</sub> for each PUA tested. For OCTA, cultures were sampled at T<sub>0</sub>, 30 min (only for OCTA 16 µM), 1 h, 2 h, 4h, and 12 h; for HEPTA cultures were sampled at T<sub>0</sub>, 4 h, 7 h and 12 h.

For ROS detection, three independent cultures were grown in 1 L glass flasks until they reached the late exponential phase of growth. Cells were harvested and directly exposed to the same concentrations of OCTA and HEPTA used for gene expression measurements and analyzed through flow cytometry. Samples were measured at T<sub>0</sub>, 20 min, 1 h, 2h and 3 h.

#### 5.2.5 Total RNA Extraction

Approximately 10<sup>7</sup> cells were collected through a filtration system using a 2 µm pore size polycarbonate filter (Nucleopore - Whatman, Maidstone, UK), over a GF/F glass microfiber filter (Whatman, Maidstone, UK). resuspended in 0.7 mL guanidine isothiocyanate buffer (RLT buffer, Qiagen, Valencia, CA, USA) containing 1% β-mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA) and immediately frozen in -80°C until total RNA extraction. After disrupting the cells by sonication (Sonicator ultrasonic processor XL, Heat System Ultrasonics, Farmingdale, NY, USA) on ice, total RNA was

isolated using the silica-membrane spin column included in the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The residual genomic DNA was removed by an additional treatment with the RNase-Free DNase Set (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. Total RNA concentration and purity were determined with a spectrophotometer (ND-100; NanoDrop Technologies, Wilmington, DE) at the wavelengths of 260 and 280 nm. DNase I-treated total RNA (1 µg) was reverse transcribed into first-strand complementary DNA (cDNA) fragments using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min. Quantitative PCRs were initiated by adding the cDNA fragments to 2× SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA), containing 250 nM of each of the forward and reverse primers. The nucleotide sequences of the primer pair used in the quantitative-reverse transcription PCR (Q-RT-PCR) were from Chung et al. (2008), and consisted of ScDSP-SG-F (5'-GAACA AGCAA ACTGC ACTCG TC-3') and ScDSP-SG-R (5'-GTCAA GAATG TTGGT CGTCG CG-3') for *ScDSP-1*. For the determination of *ScDSP-2* mRNA abundance, the primer pair used in the Q-RT-PCR was ScDSP-SG-F and a specific primer ScDSP-2-SG-R (5'-GTAGG CATCT GCTAT TCTTT CTG-3'). In addition, Ske-18S-F (5'-GAATT CCTAG ATATC GCAGT TCATC-3') and Ske-18S-R (5'-GCTAA TCCAC AATCT CGACT CCTC-3'), were used to quantify 18S rRNA. The reactions were then carried out in a GeneAmp 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). PCR settings were 95°C for 10 min, followed by 40 cycles with 1 cycle consisting of 15 s at 95°C and 1 min at 60°C for 1 min. The threshold cycle at which the fluorescence intensity exceeded a preset threshold was used to calculate the target gene mRNA and 18S rRNA expression levels. The RNA molar ratio of *ScDSP* mRNA and 18S rRNA was calculated with the formula described by Chung et

al. (2005), and the values obtained were normalized to control values and expressed as fold changes of mRNA abundance relative to control.

#### *5.2.6 Flow Cytometry*

For Flow Cytometry see Materials and Methods 2.1.2.3, Chapter 2.

## 5.3 Results

### 5.3.1 Effects of PUAs on *ScDSPs* expression

The effect of different PUAs on *ScDSP-1* and *ScDSP-2* expression was tested on *S. tropicum* for OCTA on cultures in both the early and the late exponential phase, while for HEPTA only on cultures in the early exponential phase, due to experimental limitations.

When exposed to OCTA, *S. tropicum* showed the highest *ScDSP-1* and *ScDSP-2* expression 1 h after exposure to 8  $\mu$ M (Figs. V-2, 3). The increase was higher when cells were in the late exponential phase compared to the early exponential phase (821.6 and 144.2 fold changes for *ScDSP-1* and 97.7 and 86.2 for *ScDSP-2*, respectively) (Figs. V-2 and V-3, for late and early exponential, respectively). For both *ScDSP-1* and *ScDSP-2* the highest increase in the late exponential phase was observed 1 h after exposure, with 821.6 and 97.7 times increase, respectively) (Fig. V-2 a, b). An earlier peak in DSP expression was not evident with OCTA 16  $\mu$ M at 30 min in early exponential cultures (Fig. V-3), similar to what observed with late exponential cultures and confirming that the highest increase at 1h was real and not due to a sampling time bias. The increase in both *ScDSP-1* and *ScDSP-2* expression was always lower than the expression level at 1h (Fig. V-3 a, b). When comparing expression patterns between cells in the late and the early exponential phases of growth, differently from the late exponential phase, *ScDSP-2* expression in response to OCTA in the early exponentially growing cells showed a second increase at 12h with the highest increase at 12 h for OCTA 8  $\mu$ M (80.0 fold changes) (Fig. V-3 b). Since the expression level of *ScDSP* genes was normalized to 18S rRNA, this different pattern might be due to the fact that at 12 h cells started to die, which resulted in an alteration of 18S expression, The reason why this was not evident in late exponentially growing cells might depend on the fact that in the early exponential phase cells were

more sensitive to OCTA. Another difference in the expression pattern of *ScDSP2* between the late and early exponential phases of growth is that the decrease at 2 h with respect to the peak at 1h was 79.9 % in late exponentially growing cells (Fig. V-2), while only 20.9 % in early exponentially growing cells, indicating that expression level of *DSP2* remained higher for a longer time, possibly indicating a stronger sensitivity to PUAs.

HEPTA was tested only on cultures in the early exponential phase of growth. Both for *ScDSP-1* and *ScDSP-2*, the pattern of expression levels was different from the one observed with OCTA (Fig. V-4). In particular, the highest increase in expression levels was found with HEPTA 36  $\mu$ M at 12h as compared to 1h with OCTA (489.4 and 433.3 fold changes increase for *ScDSP-1* and *ScDSP-2*, respectively). This indicates on the one hand that a higher concentration of HEPTA was necessary in order to elicit a response in both *ScDSP1* and *ScDSP2* expression, and on the other hand that a longer time of exposure gave the highest increase in both *ScDSP1* and *ScDSP2* expression. Interestingly, for *ScDSP-1*, both with 18  $\mu$ M and 36  $\mu$ M HEPTA, a first peak of increased level of expression was found at 4h (58.9 and 325.3 fold changes, respectively), followed by a decrease at 7h (9.8 and 205.9 fold changes, respectively). This pattern of expression differs substantially from *ScDSP1* pattern of expression in response to OCTA exposure (Fig. V-2 and V-3), where only one major increase was observed only at 1h, both in early and late exponentially growing cells. With HEPTA-, after 7h a new increase in *ScDSP1* expression was evident at 12h (103.8 and 489.4, respectively) (Fig. V-4 a). Additionally, differently from OCTA-exposed *S. marinoi* cells, this increase at 12h was evident both in *ScDSP1* and *ScDSP2* pattern of expression (Fig. V-4 a and b).

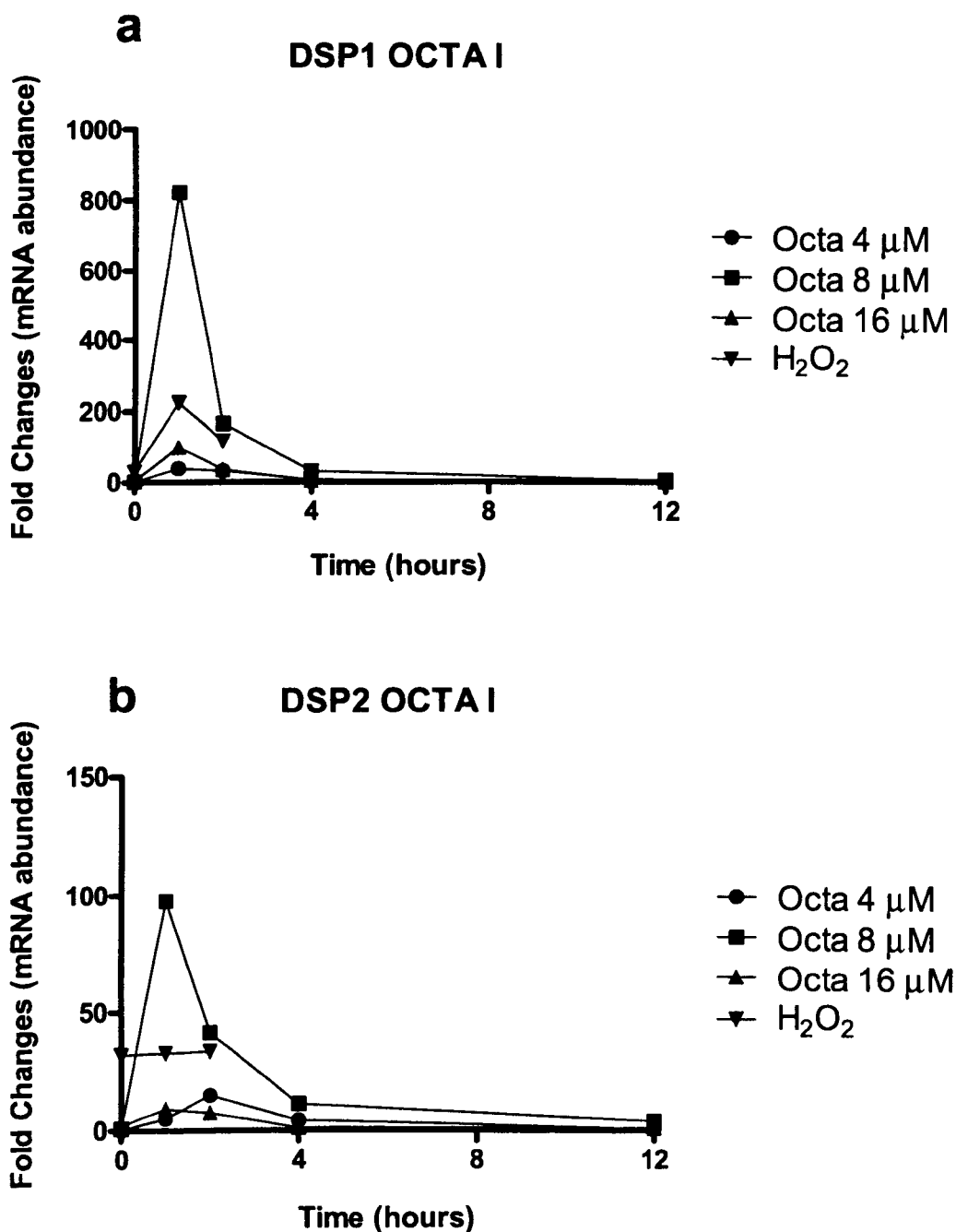


Figure V- 2: Effect of different concentrations of OCTA on *ScDSP-1* (a) and *ScDSP-2* (b) level of expression, on *S. tropicum* cells in the late exponential phase of growth. Data are expressed as mean of fold changes of mRNA abundance (relative to control)  $\pm$  standard error (SE) ( $n=3$ ), and in data point without an error bar, the error bar is smaller than the symbol.



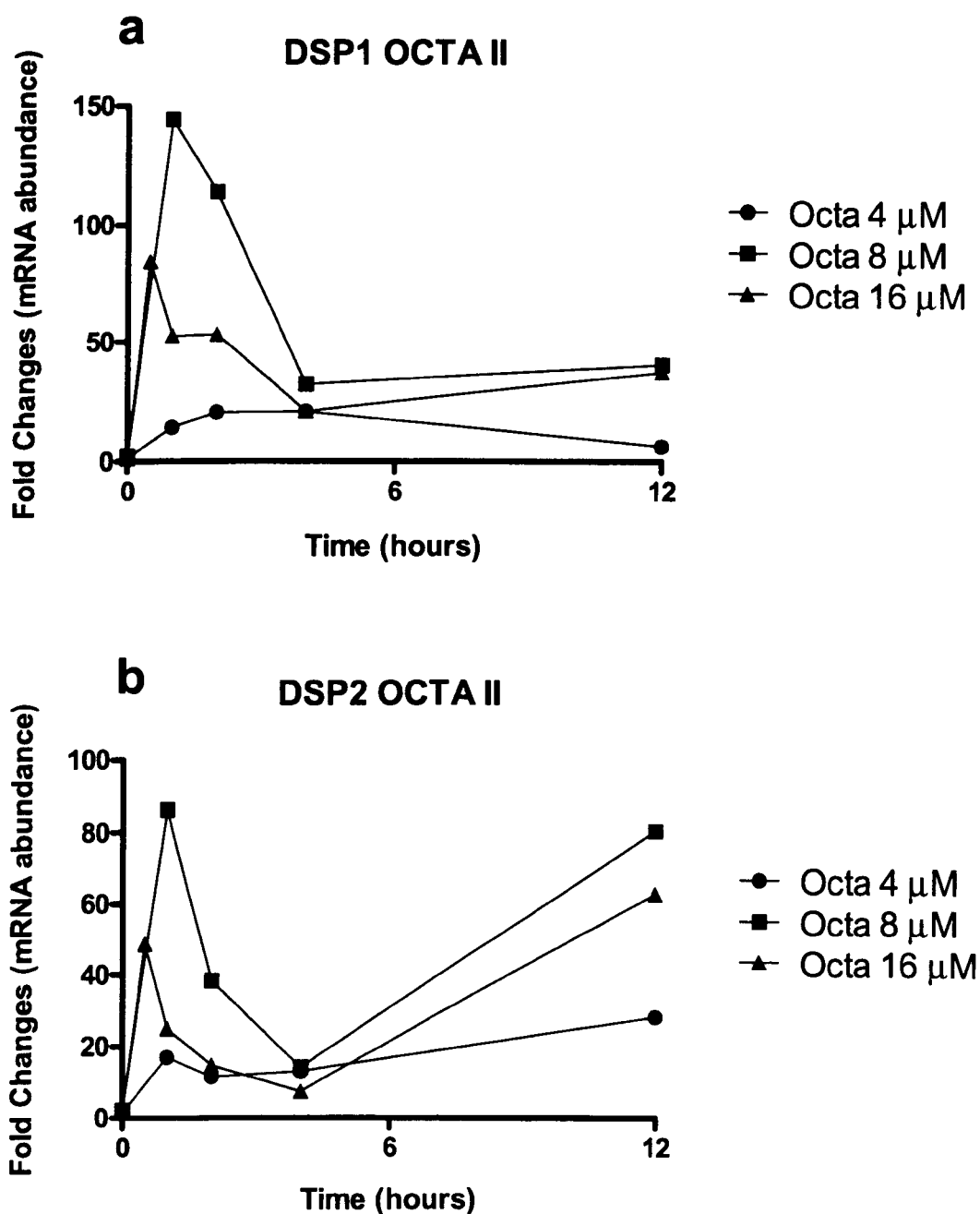


Figure V- 3: Effect of different concentrations of OCTA on *ScDSP-1* (a) and *ScDSP-2* (b) level of expression, on *S. tropicum* cells in the early exponential phase of growth. Data are expressed as mean of fold changes of mRNA abundance (relative to control)  $\pm$  standard error (SE) ( $n=3$ ), and in data point without an error bar, the error bar is smaller than the symbol.

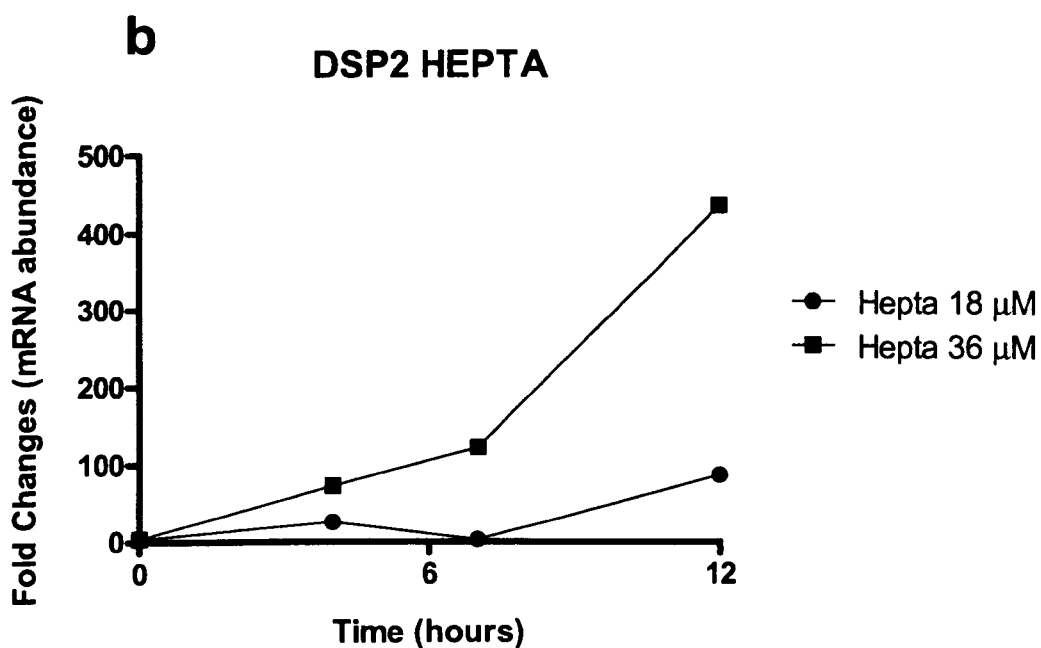
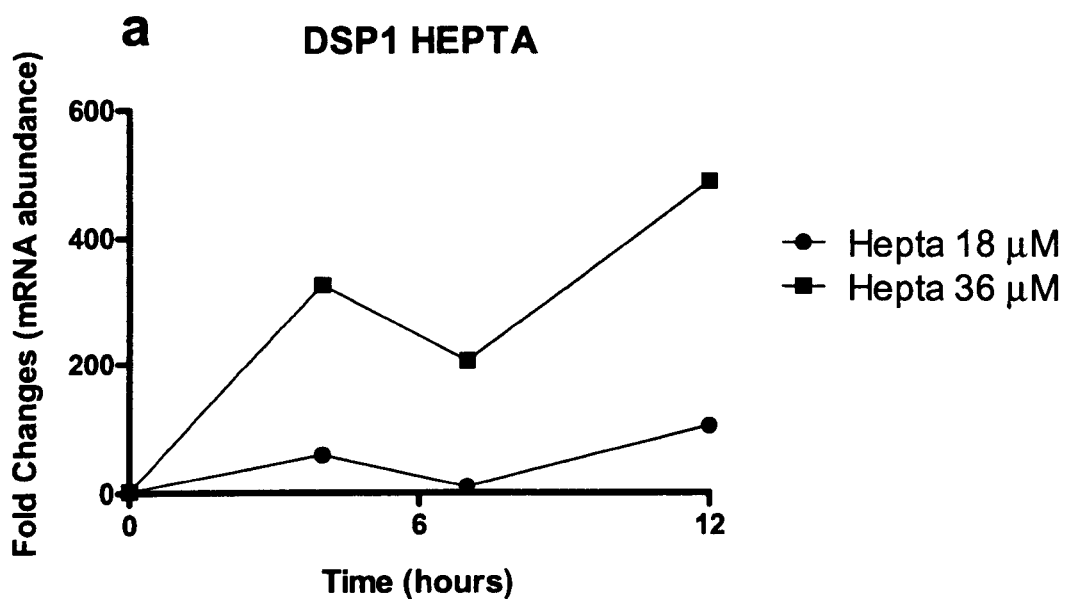


Figure V- 4: Effect of different concentrations of HEPTA on *ScDSP-1* (a) and *ScDSP-2* (b) level of expression, on *S. tropicum* cells in the early exponential phase of growth. Data are expressed as mean of fold changes of mRNA abundance (relative to control)  $\pm$  standard error (SE) ( $n=3$ ), and in data point without an error bar, the error bar is smaller than the symbol.

### 5.3.2 Growth rates

16  $\mu\text{M}$  OCTA induced the strongest decrease in growth rate ( $-0.8 \pm 0.22 \text{ d}^{-1}$ ) with respect to the control ( $0.46 \pm 0.13 \text{ d}^{-1}$ , Table V-1) in late exponentially growing cultures, equivalent to a 57.5% reduction. A similar result was observed for early-exponentially growing cultures with  $-0.67 \pm 0.00 \text{ d}^{-1}$  with respect to  $0.30 \pm 0.09 \text{ d}^{-1}$  of the control (44.78% reduction) (Table V-1). With HEPTA-exposed cells, the decrease of *S. tropicum* growth rate was similar both at 18  $\mu\text{M}$  and 36  $\mu\text{M}$  HEPTA ( $-0.24 \pm 0.33 \text{ d}^{-1}$  and  $-0.20 \pm 0.06 \text{ d}^{-1}$ , respectively) (Table V-1). Cells exposed to  $\text{H}_2\text{O}_2$ , in the late exponential phase, strongly affected cell concentrations which decreased 50% already 2 h from the inoculum (from  $2.13 \times 10^5 \pm 6.53 \times 10^4$  to  $1.02 \times 10^5 \pm 3.34 \times 10^4$ ).

Table V- 1: 24h growth rates of *S. tropicum* cultures exposed to different concentrations of OCTA and HEPTA in the late and early exponential phase of growth. Data are means of duplicates  $\pm$  SD.

Treatment	24h Growth rate (d <sup>-1</sup> )
<b>OCTA Late Exponential</b>	
Control	0.46 $\pm$ 0.13
4 $\mu$ M	0.03 $\pm$ 0.08
8 $\mu$ M	-0.63 $\pm$ 0.39
16 $\mu$ M	-0.80 $\pm$ 0.22
<b>OCTA Early Exponential</b>	
Control	0.30 $\pm$ 0.10
4 $\mu$ M	0.14 $\pm$ 0.07
8 $\mu$ M	-0.29 $\pm$ 0.09
16 $\mu$ M	-0.67 $\pm$ 0.00
<b>HEPTA Early Exponential</b>	
18 $\mu$ M	-0.24 $\pm$ 0.34
36 $\mu$ M	-0.20 $\pm$ 0.06

### 5.3.3 ROS production

When *S. tropicum* cells in the late exponential phase of growth were exposed to both OCTA and HEPTA, a dose-dependent increase in ROS production was observed, with a peak appearing within 20 min after inoculation.(Fig. V-5, a and b). With OCTA, the highest increase was observed with 16  $\mu$ M 20 min after the inoculum ( $1.9 \pm 0.2$  increase;  $p < 0.001$ ,  $n = 3$ ), while with HEPTA the highest increase occurred with 36  $\mu$ M also after 20 min ( $2.1 \pm 0.1$  increase;  $p < 0.001$ ,  $n = 3$ ). Both the ROS scavenger Tempol and the SOD inhibitor DETC induced a lower DHR-ROS green fluorescence, confirming that the observed increase was really due to ROS production (Fig. V-5 b). Additionally, the ROS donor  $H_2O_2$  was able to induce the highest increase in DHR-ROS green fluorescence 40 min after the inoculum ( $7.5 \pm 0.9$  times the control;  $p < 0.001$ ,  $n = 3$ ) (Fig.V-5 c). To confirm that the increase in ROS production was not due the solvent used to prepare PUAs, MeOH was also tested, and no increase in DHR-derived green fluorescence with respect to the control was observed (Fig.V-5 c).

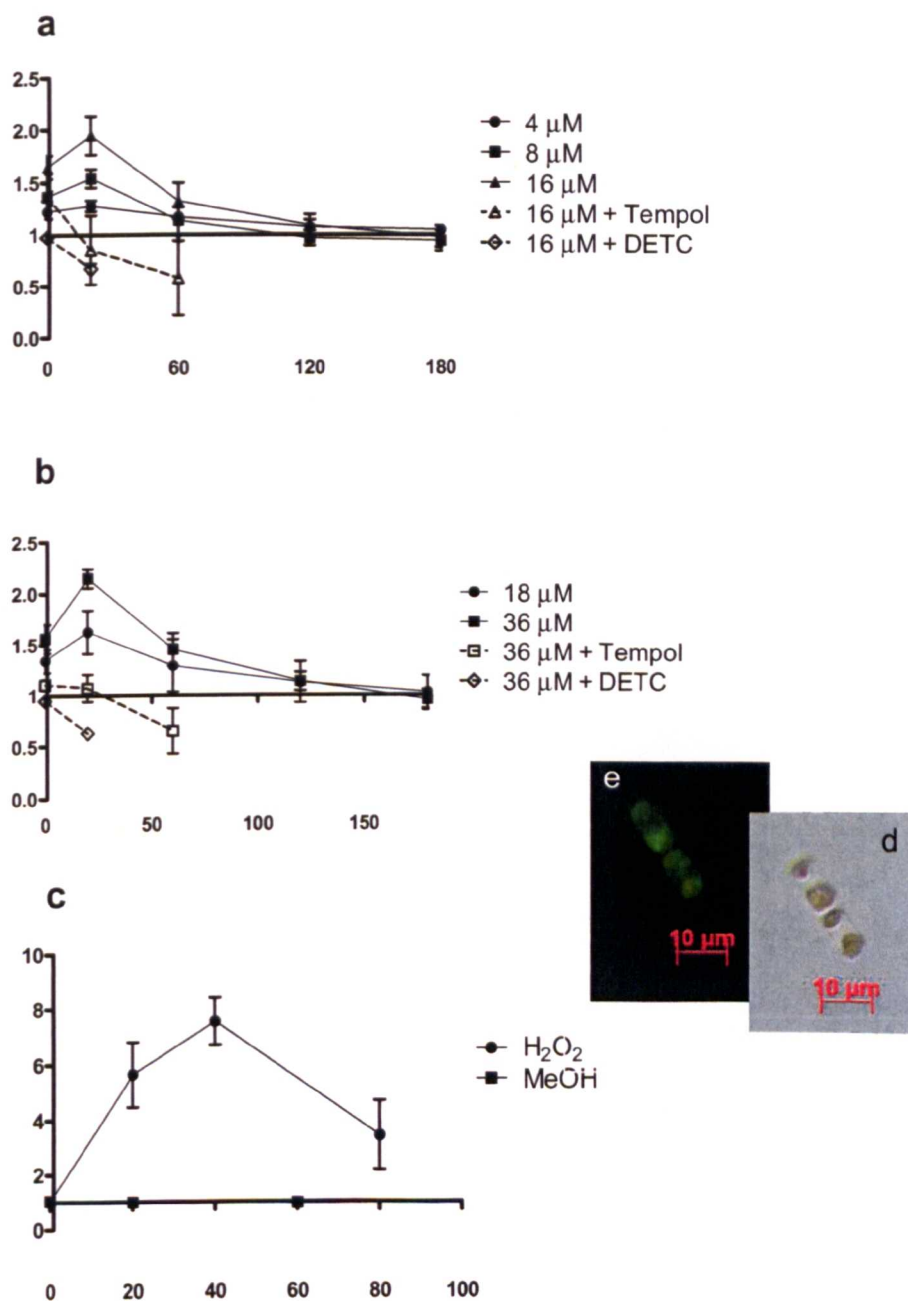


Figure V- 5: ROS production in *S. tropicum* exposed to different concentrations of different PUAs and chemicals. (a) OCTA; (b) HEPTA; (c)  $H_2O_2$  and MeOH. (d) *S. tropicum* cells observed with light microscopy and (e) green fluorescence of DHR123-loaded *S. tropicum* cells observed with epifluorescence microscopy. Data are expressed in terms of ROS-DHR123 derived green fluorescence relative to control (i.e. dye-loaded samples processed like all treatments except for PUAs inoculation). *In vivo* fluorescence was detected through flow cytometry. Data are means  $\pm$  SD from three biological replicates. Each experiment was performed at least twice. Tempol and DETC represent a ROS scavenger and a SOD inhibitor, respectively, inoculated with the highest concentration of PUA tested in each experiment.

## 5.4 Discussion

In *S. tropicum* (Kao strain) an increase in *ScDSP-1* and *ScDSP-2* expression was observed when exposed to different concentrations of PUAs, both in the early and late exponential phase of growth. In parallel, a dose-dependent increase in ROS production was also evident (Figs. V-2, 4, 6 and 8). In the same strain, an increase in *ScDSP* expression has been previously reported when cultures entered senescence (Chung et al., 2005) and also in response to light stress involving the blockage of electron flow at the level of the photosystem II (PS-II) (Chung et al., 2008). This suggests a role of DSP in mediating the molecular mechanism of auto-mortality in this diatom under stress. The same authors also reported that nitric oxide (NO) functions as a signalling molecule in the regulation of *ScDSP* expression (Chung et al., 2008). *ScDSP* has been predicted to encode a membrane-bound protein that contains a pair of EF-hand domains sensitive to changes in intracellular calcium ( $\text{Ca}^{2+}$ ) concentrations determined by external stimuli (Chung et al., 2005). When  $\text{Ca}^{2+}$  ions bind to EF-hand motifs they cause a change in the peptide conformation, which cause its activation or inactivation and triggers the signal transduction cascade (Luan et al., 2002; Means and Dedman, 1980). In metazoans, it is well known that an imbalance in the intracellular calcium homeostasis can be toxic for the cell, and it results in the activation of different calcium-regulated proteins leading to cell death, by either necrosis or apoptosis (Inbal et al., 2002; Nakagawa and Yuan, 2000; Orrenius et al., 2003; Robertson et al., 2000).

Interestingly, gene expression was maximal at the concentrations where ROS production was intermediate, suggesting that there exists a threshold for activation of these death-related genes. In other terms, since *ScDSP-1* is supposed to be involved in a PCD-type of cell death (Chung et al., 2005), it is possible to speculate that only around a specific PUA concentration a self-induced autocatalytic cell death is triggered in *S.*

*tropicum*. In particular, much lower concentrations (factor of 2x) are unable to induce such a response while much higher concentrations (factor 2x) possibly shift the type of cell death from PCD-like to necrotic (which is not genetically controlled). The latter is probably related to the excess oxidative stress induced. Possible ways to distinguish between an apoptotic vs. a necrotic type of response include the use of specific assays that test for apoptotic features of cell death, such as the cleavage of genomic DNA during apoptosis, which might yield double-stranded, low molecular weight DNA fragments (mono- and oligonucleosomes) as well as single strand breaks (“nicks”) in high molecular weight DNA (e.g. Gorczyca et al., 1993). Those DNA strand breaks can be identified by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction. The TUNEL (TdT-mediated dUTP nick end labeling) technique allows the identification of apoptotic cells through the labeling of the free 3'-OH termini of DNA strand breaks with green-fluorescing fluorescein labels incorporated in modified nucleotide polymers, which results in apoptotic nuclei expressing green fluorescence (e.g. Gold et al., 1994). In marine phytoplankton TUNEL assay has been previously used on the diatom *T. weissflogii* (Casotti et al., 2005) and the dinoflagellate *P. gatunense* (Vardi et al., 1999). Other possible techniques include the use of Annexin V, which identifies the externalization of phosphatidylserine residues (e.g. Segovia et al., 2009; Dingman et al., 2012) or DNA laddering (e.g. Affenzeller et al., 2009; Ding et al., 2012). On the contrary, typical morphological features of necrosis that could be tested by include swelling, disruption of organelle membranes, condensation of mitochondria and the formation of cytoplasmic blebs (Jimenez et al., 2009). Loss of membrane integrity, typical of necrosis, could also be tested by the use of fluorescent dyes, such as fluorescein diacetate (FDA) which will cross only damaged plasma membranes (e.g. Veldhuis et al., 2001).



In metazoans, an increase in exposure time or concentration of cell death stimulatory factors, induce a shift from apoptotic to necrotic cell death (Leist and Nicotera, 1997). For instance with glutamate, nitric oxide and ROS, it has been shown that an overstimulation leads to additional lethal reactions, which cause cell lysis prior to the completion of the PCD programme (Ankarcrona et al., 1995; Dypbukt et al., 1994; Bonfoco et al., 1995). In the raphidophyte *Heterosigma akashiwo* different levels of heat stress are able to induce a range of responses, going from recovery, to PCD-induction and finally to necrotic death when the stress is too severe (Dingman and Lawrence, 2012). With this respect, it is important to point out that, although in my experiments I do not provide evidence for the occurrence of apoptotic-like features in *S. tropicum* in response to PUAs, it has been previously reported that PUAs were able to induce apoptotic like-cell death in the marine diatom *Thalassiosira weissflogii* (Casotti et al., 2005). Moreover, in the marine diatom *P. tricornutum* overexpressing a gene associated to NO production (designated as PtNOA), PUA exposure resulted in an altered expression of superoxide dismutase and an increased metacaspase activity, which are key factors in the stress responses and cell death pathway (Vardi et al., 2008). Indeed, in my experiments, I observed a negative growth rate in OCTA-exposed *S. tropicum* cultures, both in the late and early exponential phase of growth (Table V-1), providing evidence for a toxic effect of PUAs on *S. tropicum* cultures. Interestingly, it has been recently reported that polyunsaturated fatty acids (PUFAs) have an inhibitory effect towards fungal biofilm formation of *Candida albicans* and *Candida dubliniensis* (Thibane et al., 2012). The authors reported that PUFAs were able to induce accumulation of intracellular ROS resulting in apoptotic cell death, speculating that PUFAs could function as natural antifungal compounds (Thibane et al., 2012).

Another important aspect to be considered is that in my experiments the expression level of target genes is reported as n-fold changes of *DSPs* mRNA abundance relative to the endogenous control (18S rRNA). Chung et al (2005) have previously demonstrated that for *S. tropicum* 18S rRNA is a good reference gene for the quantification of *ScDSP* expression by the absolute ratio method of quantification, which I applied in the analysis of my data. However, it is important to note that different reference genes can be used in Q-RT-PCR as endogenous control to normalize the expression of target genes of interest. The most commonly used reference genes include b-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and 18S ribosomal RNA (Huggett et al., 2005). Their use was established for non/semiquantitative techniques such as Northern blots and conventional RT-PCR assays, where a qualitative change was being measured. This was because these RNAs are expressed at relatively high levels in cells and thus they represent ideal positive controls. However, the advent of real-time PCR has introduced the issue of a re-evaluation of the use of these reference genes in light of quantitative changes. The problems associated with the selection of appropriate reference genes were discussed in a paper by Vandesompele et al. (2002), where the authors recommended using the geometric mean of multiple, carefully selected reference genes for normalization. Additionally, a recent paper has evaluated two housekeeping genes, TBP (encoding the TATA box-binding protein) and EFL (encoding the translation elongation factor-like protein), as candidates for reference genes in Q-RT-PCR assays for marine phytoplankton (Kang et al., 2012). The authors concluded that in diatoms EFL proved to be a good reference gene. Additionally, other reference genes still commonly used to normalise levels of expression in phytoplankton include the actin gene (Chong et al., 2011; Tiam et al., 2011) and the glyceraldehyde-3-phosphate dehydrogenase gene (Lavaud et al., 2012).

ROS are generally indicated as PCD-inducing factors (Simon et al., 2000; Aldsworth et al., 1999). However, it is suggested that a difference exists between the intracellular effectors of a PCD-like process (e.g. NO and ROS), and the actual inducing factors and stimuli causing an alteration of such intracellular mediators possibly leading to PCD-like processes or, alternatively, non-PCD-related death processes. Based on the results reported here, what is proposed in the case of *S. tropicum*, is that PUAs might act as (external) inducing factors, causing a modification in the intracellular ROS level, which would result in a differential activation of *ScDSP*, depending on the amount of ROS produced and possibly on the cell physiological and growth status.

It is possible to argue that the reason why the highest OCTA concentration tested (i.e. 16  $\mu$ M) was not found to induce the highest increase in the level of DSP expression, despite the higher amount of ROS produced, was that the time interval for DSP expression sampling was too long, and that the increase in DSP expression might have been missed. Although, when a shorter time point was added (30 min) the expression level was not higher than at 1 h with 8  $\mu$ M OCTA (Fig. V-3 a). Considering that a burst in ROS production always occurred 20 min after exposure with all the different PUAs concentrations tested, it is possible to conclude that the highest level of DSP expression with 8  $\mu$ M OCTA was indeed due to the specific PUA concentration and associated ROS levels, rather than to an experimental bias.

It had been previously observed that H<sub>2</sub>O<sub>2</sub> is able to significantly induce *ScDSP* expression in *S. tropicum* cells (Chung, pers. comm.). It is interesting to note that in the experiments reported here H<sub>2</sub>O<sub>2</sub> did not induce the highest DSP expression, even though it elicited the overall highest increase in ROS-DHR derived green fluorescence ( $7.5 \pm 0.9$  times the control at 40 min after exposure, Fig. V-6 c). ROS-concentration dependent cellular responses have been reported to range from up-regulation of antioxidant defence

to cell death by necrosis (Chandra et al., 2000), with intermediate concentrations inducing PCD or apoptosis (Korsmeyer et al., 1995; Levine et al., 1996). In the dinoflagellate *P. gatunense*, it has been observed that high H<sub>2</sub>O<sub>2</sub> levels (i.e. higher than 5 mM), resulted in necrotic cell death with a significant decline in cell numbers, as opposite to lower H<sub>2</sub>O<sub>2</sub> concentrations which induced an increase in metacaspase transcript abundance, thus suggesting for an involvement of H<sub>2</sub>O<sub>2</sub> in triggering apoptotic cell death (Murik and Kaplan, 2009).

Chung et al. (2005, 2008) reported that the increase in *ScDSP-1* mRNA levels in *S. tropicum* ageing cells was strongly responsive to NO production, thus proposing that *ScDSP-1* expression is mediated by NO signalling. Based on the results reported here, it is hypothesized that in a stress-type of response, such as exposure to PUAs, DSP expression is instead regulated by ROS signalling. However, it must be taken into account that in my experiments I did not test for NO production upon exposure to PUAs in *S. tropicum*, which could represent a future line of research. Moreover, given that DSP has been suggested to be a Ca<sup>2+</sup> regulated protein possibly involved in an apoptosis-like process (Chung et al., 2005), it is important to point out that both NO and ROS are also known to act as Ca<sup>2+</sup> mobilizing agents in different biological systems (Gonzalez et al., 2002; Mallilankaraman et al., 2011; Wendehenne et al., 2004; Neill et al., 2003). It is suggested here that based on the type of stress factor, an intracellular imbalance of different reactive molecules might be induced (i.e. reactive nitrogen species or reactive oxygen species). This, in turn, might induce an increase in *DSP* expression and possibly also Ca<sup>2+</sup> mobilization, with subsequent regulation of DSP activity.

With respect to ROS signalling in response to PUAs exposure, it is worth noting that in the congeneric PUA-producing *S. marinoi* I previously reported that increased ROS levels induced an enhanced production of the xanthophyll diatoxanthin (Dt), which was

likely to have antioxidant properties (see Chapter 3). Based on the findings reported here it is possible to speculate that in *Skeletonema* spp. the ROS downstream response to PUAs may activate either a protection pathways (i.e. antioxidant defence) or a cell death cascade through activation of specific genes, depending on the PUA concentration, the time of exposure, the concentration of ROS produced and the physiological state of the cell. This is likely to indicate that ROS may have an important function in the molecular cascade following PUAs exposure and this has important implications for population dynamics and cell lysis, as instance, during diatom blooms. In the natural environment, cell lysis has been reported to reach its maximum at the end of phytoplankton blooms (Vanboekel et al., 1992). It is known that this process can be induced both by exogenous factors, such as bacteria, viruses and nutrient limitation (Agusti and Duarte, 2000), as well as endogenous factors such as cell damage, senescence, and genetically auto-induced mortality (Veldhuis et al., 2001). Additionally, it has also been suggested that PUAs might be expected to be released in seawater after cell lysis, thereby affecting organisms in the surrounding medium (Casotti et al., 2005). Interestingly, it has been proposed that the evolution of PCD in unicellular organisms as a byproduct of selection might be explained by the possibility that PCD could be a maladaptive stress-induced process that could be selected under certain conditions for specific group benefits (Nedelcu et al., 2010). In relation to this idea, an interesting concept proposed is that if a intrapopulation signal (e.g. secondary metabolites, such as PUAs) stimulate the ancestral PCD-inducing signal (e.g. ROS), and if this intrapopulation signal-dependent death is beneficial for the population (as it might be the case in a bloom termination event) this type of PCD could be selected for and consequently evolve into an altruistic adaptation (Nedelcu et al., 2010).

In *S. tropicum* the levels of *ScDSP* expression in response to OCTA was investigated both in cells in the early and the late exponential phase of growth. A different level in the increase of the expression was observed in the two stages of the exponential phase of growth, with a higher induction occurring in the late exponential phase of growth. These results well correlate with the finding that *DSP* expression increases in ageing cells, and in particular reaches its maximum when cells enter the decay phase (Chung et al., 2005).

Chung et al. (2008) reported that the gene *ScDSP-2* was found to be located upstream of *ScDSP-1* start codon, and its putative amino acid sequence was found to have a 73.7% identity with that of *ScDSP-1*. In the experiments reported here *ScDSP-1* and *ScDSP-2* in OCTA-exposed late exponential cells displayed a similar expression pattern, with *ScDSP-1* expression being higher in comparison to *ScDSP-2* (Fig. V-2). When comparing *ScDSP-1* expression between OCTA-exposed late and early exponential cells, still a similar pattern of expression was found, with, however, a higher increase in *ScDSP-1* expression in the late exponential phase in comparison to the early exponential phase, likely to be related to intrinsic differential levels of *DSP* expression in different growth stages (Chung et al., 2005). However, when looking at *ScDSP-2* expression between OCTA-exposed cells in the late and early exponential phase of growth, a different pattern of expression was observed (Fig. V-2 b and Fig. V-4 b, respectively). In particular, 12 h after exposure, an increase in *ScDSP-2* expression in OCTA-exposed cells in the early exponential phase of growth was observed, comparable to the expression level after 1h of exposure, with all the OCTA concentrations tested (Fig. V-3 b). An increase in *ScDSP-2* expression after 12 h was also evident in HEPTA-exposed cells in the early exponential phase of growth (Fig. V-4 b). It might be possible that different patterns of expression in *ScDSP-2* with OCTA and HEPTA in early exponential growth cells are due to the fact that this gene could not directly be involved in an apoptotic type of signalling, but rather

in either a general cell death process (not related to PCD), or an alternative, non-apoptotic PCD mechanism. Indeed, in the chlorophyte *Dunaliella viridis*, different types of cell death might occur depending on the intensity and the nature of the stimulus, and apoptosis and necrosis might represent only two extremes of a continuum of intermediate forms of cell death (Jimenez et al., 2009).

*S. tropicum* cells in the early exponential phase of growth were also exposed to HEPTA. In this case, the pattern of *ScDSP-1* and *ScDSP-2* expression was different as compared to OCTA. With both genes the highest increase in the level of expression was found 12 h after exposure (Fig.V-4). Differently from OCTA, cells exposed to HEPTA showed the highest increase of both *ScDSP-1* and *ScDSP-2* expression at the highest concentration (36  $\mu$ M) after 12 h, which was also the concentration at which the highest increase in ROS production was observed after 20 min (Fig. V-6 b). However, it must be noted that different time intervals were selected with respect to OCTA, and that the first sample was taken 4 h after the inoculum (Fig.V-4). It is possible to speculate that at 4 h and 12 h the observed increase in DSP expression was the result of different processes not directly related to HEPTA toxicity. Possibly, the high level of ROS induced after 20 min of exposure (Fig. V-6 b) induced some other different pathways (e.g. necrosis), leading to a secondary peak 12 h later. This would be consistent with an involvement of *ScDSP-2* in an alternative cell death process differently from *ScDSP-1* (as previously suggested in this discussion).

An interesting aspect related to PUAs putative function of inducers and regulators of PCD-like processes in diatoms, is that phytoplankton species using such chemical signals may have been selected during the course of evolution due to their better ecological success. This could also explain the widespread occurrence and strong ecological success of different *Skeletonema* species in the natural environment (Kooistra et al., 2006;

Kooistra et al., 2008). It could also be speculated that those species able to produce PUAs or different bioactive metabolites, and therefore capable of using them as chemical cues, may have an ecological advantage compared to non-producing ones, both within the same genus but also among different diatom genera. It is worth noting that Cervia et al. (2009) reported that the secondary metabolite Euplotin C derived from the marine ciliated protist *Euplotes crassus*, was found to induce PCD in the congeneric *Euplotes vannus*, which does not produce this metabolite. The authors suggested that specific secondary metabolites can play an important ecological role in broadening phytoplankton niche size through different mechanisms, including for instance autocatalytic induced cell death (Cervia et al., 2009). This could also be the case for the PUA-producing diatoms.

The *S. tropicum* strain used in this study has been confirmed to produce oxylipins, the family of bioactive metabolites derived from the oxidative transformation of fatty acids (Pohnert and Boland, 2002; Moore, 1999), even though specific PUAs production has not been reported so far (d'Ippolito, pers. comm.). However, the production of such metabolites is reported to vary considerably between strains and clones of the same species (Taylor et al., 2009; Gerecht et al., 2011). In addition, recent evidences point to additional diatom-derived cytotoxic compounds other than PUAs, produced through the same biosynthetic pathways (Ianora and Miralto, 2010). These compounds include hydroxyacids (HEPEs) and epoxyalcohols (HepETEs) (Ianora et al., 2011b; Barreiro et al., 2011). In this respect, what would be highly relevant from an ecological point of view is to test the possible involvement of *ScDSP* (or *ScDSP*-like) gene expression in the well-known PUA-producing species *S. marinoi*, which could be an interesting aspect for future research.



# CHAPTER 6

## Conclusions

In recent years, chemical ecology has drawn much attention in the scientific community due to the increasing evidence that chemical cues play a determining role in regulating biotic interactions and therefore shaping plankton communities biodiversity and functioning (Ianora et al., 2011a). Among these, diatom-derived secondary metabolites such as polyunsaturated aldehydes (PUAs) play different and simultaneous functions, including an indirect antipredatory role against copepod grazing (Ianora et al., 2004; Miralto et al., 1999), allelopathic functions against other phytoplankton species (Ribalet and Casotti, 2006), a role as determinants of the structure of marine bacterial communities (Ribalet et al., 2008; Balestra et al., 2011), as well as a function as cell signals within diatom populations (Vardi et al., 2006; Casotti et al., 2005).

The main aim of this work was to better examine the effects of PUAs on the PUA-producing species *Skeletonema marinoi* and in the congeneric *S. tropicum*, trying to relate the observed response to possible dynamics occurring at sea and to the potential role of these secondary metabolites as both allelo- and infochemicals in diatom populations. In particular, this work focuses on nitric oxide (NO) and reactive oxygen species (ROS) dynamics and on their potential effects at the cellular and molecular levels in response to PUAs.

Nitric oxide (NO) production in *S. marinoi* was initially investigated during standard growth conditions, in order to better understand the physiological role of NO during optimal growth (Chapter 2). As previously reported in different phytoplankton species (Zhang et al., 2006b; Estevez and Puntarulo, 2005), also in *S. marinoi* NO

showed to have an important role in determining key passages during the life of a culture, suggesting a role as growth-related factor, with relevant implications for studies of phytoplankton growth dynamics also in the field. NO has been suggested to function as both an early indicator (Tang et al., 2011) and a stimulator (Zhang et al., 2005) of cyanobacteria and diatom blooms, respectively. Considering that *S. marinoi* is a cosmopolitan bloom-forming species (Kooistra et al., 2008), a potential role for NO as an indicator of growth status and its involvement in regulating cell density during natural blooming conditions is a promising aspect for future research.

An important finding of this study is that in *S. marinoi* a nitrate reductase (NR)-dependent biosynthetic pathway is likely to be active during the log phase of exponential growth (Chapter 2), while a nitric oxide synthase (NOS)-like enzyme might be involved in other growth phase. When exposed to the PUA OCTA and specific inhibitors, *S. marinoi* showed a different response, suggesting the presence of both a NOS and a NR pathway (Chapter 4), probably dependent on the physiological vs. stress conditions. This suggests that a NOS-like biosynthetic pathway is active only under specific (optimal) cellular conditions.

In PUA-exposed *S. marinoi* cells it was not possible to detect an increase in NO production (Chapter 3.1). This finding was surprising as NO has been reported to be involved in stress responses in many marine macro-and microalgae (Zhang et al., 2006b; Bouchard and Yamasaki, 2009; Ross et al., 2006), including other diatoms (Vardi et al., 2006). The non-PUA producing diatom *Phaeodactylum tricornutum* responds to the PUA DECA with an increase in NO (Vardi et al., 2006), indicating that different stress-signalling pathways are elicited by this PUA in the two species. This finding suggests a species-specific type of response to PUAs exposure, possibly related to the ability to produce PUAs and thus to encounter them in the natural environment. Another

interesting aspect is that while *S. marinoi* does not produce NO in response to any PUA, *P. tricornutum* does so only for DECA and not for the other PUA tested (see Chapter 3.1, Fig.III-5 b), which indicates also the existence of a PUA-specific type of response. It might be possible that the response elicited by DECA in *P. tricornutum* is related to the compound toxicity, while the response to OCTA is related to its natural occurrence and associated higher probability of encounter it at sea. This reinforces the hypothesis that commonly present PUAs such as OCTA and HEPTA may act as infochemicals. However, despite being the most used PUA in toxicological experiments, DECA is not the most common PUA present in marine phytoplankton (Wichard et al., 2005a; Vidoudez et al., 2011b). From an evolutionary perspective, it appears reasonable to consider that only molecules that are more widely present in nature have contributed to the evolution of specific response mechanisms such as signalling and allelochemistry both in PUA and non-PUA producing species.

An important point is that there was no increase in NO in response to PUA exposure, but indeed a decrease in NO-related fluorescence was found in both *S. marinoi* and *P. tricornutum* (except for DECA). On the one hand, considering that PUAs are known growth-inhibitors (Casotti et al., 2005; Ribalet et al., 2007a), and that NO has been shown to have a growth-related function in different phytoplankton species (Zhang et al., 2006b; Estevez and Puntarulo, 2005 and data presented in this thesis, see Chapter 2), the observed NO decrease might be due to the growth-inhibiting effect of PUAs (Casotti et al., 2005). On the other hand, the observed decrease might also be due to the observed shift towards the production of different nitrogen (or oxygen) reactive species. From the results from different scavengers and inhibitors of both ROS and RNS, it was evident that such a decrease in NO was related to its reaction with excess superoxide anion ( $O_2^{\cdot-}$ ), leading to peroxynitrite formation ( $ONOO^{\cdot-}$ ) (Chapter 4).

Overall, in terms of the oxidative stress response to PUAs in *S. marinoi*, it is possible to hypothesize a contribution of both  $\text{ONOO}^-$  as well as  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ -driven processes, with a major involvement of  $\text{H}_2\text{O}_2$  and  $\text{H}_2\text{O}_2$ -derived species (Chapter 4).

Finally, it was shown that light stress, in terms of the blockage of the electron flow at the level of photosystem II by the use of the photoinhibitor DCMU, elicited, instead, an increase in NO production (Chapter 3.1). This indicates a stress-specific response in *S. marinoi* involving different molecules and this is highly relevant in interpreting cell behaviour *in situ*.

In PUA-exposed *S. marinoi* cells, there appears to be a threshold for ROS production, below which no ROS are produced (Chapter 3.2). This agrees with what was reported by Vardi et al. (2006) in *P. tricornutum*, that a threshold-like response exists in cells sensing a PUA-derived stress. Interestingly, the observed response in ROS production does not appear to be related only to the toxicity of PUAs but rather to the specificity of the PUAs that are produced by the cell itself. In fact, DECA, which is not produced by *S. marinoi*, did not elicit any increase in ROS production. It is possible to speculate that this type of response is related to the involvement of selective receptors able to recognize specific PUAs. Given that an excess production of  $\text{O}_2^-$  is linked to the generation of downstream ROS/RNS and ROS/RNS-mediated effects, a key point is represented by the possible intracellular mechanisms of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  generation.

The ROS downstream response to PUAs is likely to activate genes involved in either signalling and protection or a cell death cascade, probably depending on the PUA concentration. ROS may have an important role in the molecular cascade following PUAs exposure also in nature, as instance, during final stages of diatom blooms, when cell lysis increases and PUAs are released in the surrounding medium. The data on increased diatoxanthin production triggered by PUAs supports the role of this molecule

as an antioxidant, aimed at protecting the cell from oxidative stress thereby allowing the cell to cope with stress by maintaining photosynthetic performance unaltered (Chapter 3.2). In particular, I observed a PUA concentration-dependent xanthophyll-cycle activation with faster dynamics at the highest OCTA concentrations tested (Chapter 3.2, Fig. III-10 b). Moreover, cells were able to maintain their capacity to photosynthesize, and after 24 h of exposure cells were able to recover after PUAs were removed from the medium. This suggests that ROS are the trigger of a protective response in *S. marinoi* which uses, indeed, the xanthophyll cycle during a shorter time scale, and  $\beta$ -carotene production on the long term (Chapter 3.2). It is possible to speculate that a differential type of response (i.e. protection vs. cell death) might also be present at sea during a bloom condition, when local PUA concentrations are expected to be high (Vidoudez et al., 2011a). The scenario proposed is that after PUA are released, depending on both the PUA concentration and the time of exposure, *S. marinoi* cells could either experience ROS-mediated protection and/or apoptotic cell death, as seen for DECA-exposed *T. weissflogii* (Casotti et al., 2005). It is known that PUAs are released upon cell lysis as well as during the late stationary phase of growth by intact *S. marinoi* cells (Vidoudez and Pohnert, 2008). This is of particular significance in terms of PUA dynamics at sea during the termination of a bloom event, when lysis rate is expected to be high. This also implies that the cellular response of a PUA-producing diatom to PUAs could be finely modulated by and highly dependent on the time and dose of exposure to PUAs themselves, in terms of ROS production, antioxidant defence and cell death. For those cells where a protective type of response rather than cell death is elicited, there will probably be a slowing down of the growth rate paid off by an unaltered ability to photosynthesize. This is expected to allow cells to quickly recover when the chemical

stress will be no longer present in the environment, allowing cells to restore the original population size, as observed in this study.

The dinoflagellate *Scrippsiella trochoidea* was found to form temporary cysts in response to the exposure of low concentrations of allelochemicals released by three toxic microalgae, whereas higher concentrations induced cell death (Fistarol et al., 2004). It is intriguing to speculate that something similar might also occur in *S. marinoi* during the end of a bloom at sea, where PUAs might act as infochemicals within the same diatom population by inducing cyst formation. The fact that in the experiments reported here no cyst formation was detected might be dependent on the relatively short time scale that has been investigated. Interestingly, resting stages of the congeneric *S. costatum* were found to survive for several decades in the sediments of a Swedish fjord (McQuoid et al., 2002). This is of particular relevance from an evolutionary perspective. In fact it is believed that chemically-mediated interactions might have driven the evolution of some organisms by selecting those individuals that had the ability to either resist to, exploit, or avoid external metabolites from neighbouring cells (Lucas, 1947). It has also been claimed that this could only be expected if chemical-mediated interactions were sporadic, such as during blooming events (Lewis, 1986). A similar mechanism has been also hypothesized by Ribalet (2007), to explain the periodical occurrence of PUA-producing diatoms. Such a theoretical model could also be extended by considering that among all PUA-forming *S. marinoi* cells, PUAs might be able to induce protection through antioxidant defence systems activation (see Chapter 3), possibly leading to cyst formation only in those cells that are in the best conditions to survive. In the marine dinoflagellate *Peridinium gatunense* it has been previously hypothesized that only the best adapted individuals will encyst, while the less fitted members of the population will

be eliminated through PCD, thus conferring a selective advantage to the group of individuals as a whole (Vardi et al., 1999).

In the congeneric marine diatom *S. tropicum*, PUAs were able to induce the expression of a gene encoding for a death specific protein (DSP), named *ScDSP* (see Chapter 5), thought to be involved in an apoptotic-like type of cell death (Chung et al., 2008; Chung et al., 2005). *ScDSP* expression was also found to be related to ROS production (Chapter 5). Only specific PUAs concentrations were able to elicit a specific pathway of *ScDSP* expression, consistent with a signalling role of PUAs. Interestingly, these were not the highest levels tested and were observed to elicit the production of intermediate levels of ROS (Chapter 5 Figs.V-2 and V-5), whereas lower and higher ROS levels failed to induce an increase in *ScDSP* expression. As a consequence, it appears that only specific PUAs concentrations are sensed and are able to trigger a self-induced autocatalytic cell death pathway, whereas lower concentrations are not perceived and higher concentrations shift the type of response from PCD-like to necrotic (which is not genetically controlled) cell death, due to the complete impairment of the cell's ability to cope with the excessive oxidative stress induced. Therefore, in *Skeletonema* spp. the ROS downstream response to PUAs may activate either protection pathways (i.e. antioxidant defence) or cell death through activation of specific genes, depending on the PUA concentration, the time of exposure, the concentration of ROS produced and the physiological state of the cell. This clearly suggests that ROS are key components of the molecular cascade triggered by PUA exposure. Table VI-1 schematically summarises the main highlights presented in this thesis divided by chapters.

Table VI- 1: Summary of the main findings reported in this thesis, organized by chapters

	Highlights
Chapter 2	<ul style="list-style-type: none"><li>• In <i>S. marinoi</i> NO is produced under standard growth conditions</li><li>• NO appears to have an important physiological role as a growth-regulator factor</li><li>• NO biosynthesis is likely to be dependent on nitrate reductase (NR) activity</li></ul>
Chapter 3	<p>a. NO production in response to PUAs appears to be:</p> <ul style="list-style-type: none"><li>• Stress-specific</li><li>• Species-specific</li><li>• PUA-specific</li></ul> <p>b. PUAs elicit a decrease in NO levels in <i>S. marinoi</i> which is likely to be due to:</p> <ul style="list-style-type: none"><li>• Impairment of NO production</li><li>• Shift towards PUA-specific ROS production</li></ul> <p>c. Activation of the xanthophyll cycle (XC) as a means of antioxidant protection in response to PUA-induced ROS production in <i>S. marinoi</i>.</p>
Chapter 4	<ul style="list-style-type: none"><li>• The oxidation of the ROS-sensitive dye DHR123 is likely to be dependent both on a peroxynitrite (ONOO<sup>-</sup>)- and a hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-driven process</li><li>• The decrease in NO in <i>S. marinoi</i> cells in response to PUAs exposure might be related to consumption of NO due to its reaction with excess superoxide anion (O<sub>2</sub><sup>-</sup>)</li></ul>
Chapter 5	<ul style="list-style-type: none"><li>• In <i>S. tropicum</i> PUAs induce the expression of a gene encoding for a death specific protein (<i>ScDSP</i>)</li><li>• <i>ScDSP</i> expression in <i>S. tropicum</i> has been found to be related to an increase in ROS production</li><li>• Only specific PUAs concentrations are able to elicit a specific pathway of <i>ScDSP</i> expression, likely to indicate a signalling role of PUAs</li></ul>



In the field of chemical ecology an important aspect to be taken into consideration is the ecological relevance of the concentrations used in laboratory studies, and how they relate to naturally occurring and ecologically relevant concentrations. In the case of PUAs, only a few studies have reported PUA production of natural plankton communities after cell disruption (Poulet et al., 2006; Poulet et al., 2007; Wichard et al., 2008). Two recent studies have addressed the important issue of the need to measure and estimate PUAs concentrations directly at sea (Vidoudez et al., 2011a; Vidoudez et al., 2011b). In a field study in the Adriatic Sea (Italy), Vidoudez et al. (2011a) reported the patchy distribution of PUAs associated with a spring bloom of the diatom *S. marinoi*. In particular, particulate PUAs (i.e. produced after cell disruption) and dissolved PUAs (i.e. present in the water) were measured (Vidoudez et al., 2011a). In terms of dissolved PUAs, their occurrence in natural seawater samples was reported for the first time, and *S. marinoi* was found to be the major contributor to the total PUAs detected. Dissolved HEPTA and OCTA concentrations were found to be in the nanomolar (nM) range (Vidoudez et al., 2011a). Even though these concentrations are considerably lower compared to the ones I applied in my experiments (which were in the micromolar range), it is proposed that during conditions of high cell lysis, such as during bloom decay, an increased depletion of particulate PUAs would result in an increased concentration of dissolved PUAs (Vidoudez et al., 2011a). Additionally, the authors pointed out that the reported measurement averaged over a quite large volume of seawater, while local concentrations in the vicinity of the single cells are expected to be higher due to the low diffusion process away from the producer (Ribalet et al., 2007a). In another study, Vidoudez et al. (2011b) performed a survey of the production of PUAs in manipulated mesocosms inoculated with different densities of *S. marinoi*. The authors found that *S. marinoi* was the major source for HEPTA and OCTA during the

entire bloom development and, similarly to what reported by Vidoudez et al. (2011a), they also found dissolved PUAs concentrations to be in the nanomolar range, and that their concentrations were highly variable, probably due to interfering biological material present in the mesocosm, which might have partially absorbed the released PUAs.

The ability of a certain species to produce and consequently respond to PUAs activating specific pathways is a key point to consider when analyzing bloom dynamics at sea. *S. marinoi* is a well-known PUA-producing species (Wichard et al., 2005a; Ribalet et al., 2009), while *S. tropicum* (Kao strain) so far has been reported to be able to produce oxylipins but not PUAs (d'Ippolito, pers. comm.). This is of particular relevance because different phytoplankton species are known to produce oxylipins, but only a few have been reported to produce PUAs (Ianora et al., 2011a). If a role of PUAs as allelochemicals is hypothesized (Ribalet et al., 2007a), then it is possible to speculate that the ability to produce PUAs is an evolutionary determinant, possibly conferring an advantage to those species able to produce such compounds. What could be the case in the instance of a bloom termination event, is that, after being released into seawater, PUAs can function as allelochemicals (i.e. interspecific function), inhibiting the growth of nearby species or killing them through PCD, therefore exploiting the little resources available. In parallel to this, PUAs may act as signals to select only for clones that are better adapted for producing PUAs and that possibly have evolved a more efficient way to respond to PUAs themselves. Indeed, strain and clone-specific differences in PUAs and oxylipin production have been reported in the PUA-producing diatom *S. marinoi* (Taylor et al., 2009; Gerecht et al., 2011) suggesting that such diversity confers a selective advantage to certain clones, consequently shaping diatom population dynamics (Gerecht et al., 2011). Interestingly, an early study showed that blooms of the diatom *Skeletonema costatum* had 'distinct and prevalent' forms, which could support this

hypothesis (Murphy, 1978). On the other hand, recent studies addressing the genetic structure of phytoplankton blooms indicate that they are heterogeneous, with different clonal lines contributing to population structure (Ryneckson and Armbrust, 2005; Medlin et al., 2000).

An interesting concept deriving from the previous considerations is that if a signal (e.g. PUAs) is able to stimulate a PCD-inducing signal (e.g. ROS), and if the consequent death has a positive effect on the whole population (as might be the case during final bloom stages), PCD could be selected for and consequently be maintained as an altruistic adaptation (Nedelcu et al., 2010). As a consequence, phytoplankton species characterized by this type of chemical signal might have been selected during the course of evolution due to their better ecological success. This could also explain the widespread occurrence and strong ecological success of different *Skeletonema* species in the natural environment (Kooistra et al., 2006; Kooistra et al., 2008). In this respect, it is also intriguing to refer to the controversy raised about the role of PUAs (Flynn and Irigoien, 2009). These authors suggest for a role of PUAs as either direct feeding deterrents against microzooplankton or simply as secondary metabolites without any functional defence role, claiming that an insidious type of defence against copepods would be counterproductive for diatoms themselves from an evolutionary point of view (i.e. direct vs. indirect chemical defence). Indeed, if a role for PUAs as direct allelopathic signals and infochemicals is hypothesized, as is proposed here, then it might appear even more logical that in evolutionary terms PUA-producing species would have been selected for with respect to non-PUA producing ones. What might be possible is that a role of PUAs as teratogenic compounds could be a trait derived from different evolutionary forces that acted by selecting PUA-producing species in the first place.

Future lines of research should focus on better elucidating the link between signal or stress molecules induced by PUA exposure in the well-known PUA-producer *S.marinoi* (e.g. ROS) to the possible induction of a cell death cascade, involving the expression of different genes, such as *DSP* genes, for which specific primers should be re-designed for *S. marinoi*. An intriguing future approach could also be the use of these genes to measure their expression levels as targets of specific bloom stages (i.e. the decay of a bloom) directly in the field. Moreover another possible approach that could be tested in terms of specific RNS and ROS involved in the stress response of *S. marinoi* to PUAs could be to look at the enzymatic activity of specific enzymes which are responsible for the ROS or RNS thought to be involved in the response. Additionally, testing NO production in *S. tropicum* remains to be validated in order to possibly extend the link between *DSPs* expression and NO production, as this has been previously demonstrated for photoinhibition-derived stress (Chung et al., 2008).

In conclusion, from an ecological point of view the findings reported here suggest that diatom species are able to perceive and discriminate the different PUAs they are exposed to, probably based upon their life history. It is suggested that the interactions between chemical signals and reactive pathways underlie the functional diversity of species and their ability to cope with the environment. Indeed, the physiological responses to stimuli and biological interactions are interconnected and can shape the ecosystems in a dynamical way, determining the ecological success of a species and its role.

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